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Applicant : David Berd
Serial No. : 08/203,004
Filed : February 28, 1994
For : **COMPOSITION AND METHOD OF USING TUMOR CELLS**
Attorney : PFF:AL:bar
File No. : **1225/0C674**

Mailed : April 9, 2002 Mailer: AD

#33

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March 13, 2003

Reference: 1225/0C674

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VIA FEDERAL EXPRESS

Examiner Susan Ungar
Art Unit: 1642
Crystal Mall 1, 7th Floor
1911 South Clark Street
Arlington, VA 22202

Re: **U.S. Patent Application Serial No. 08/203,004**
For: COMPOSITION AND METHOD OF USING TUMOR CELLS
Attorney Docket No. 1225/0C674

Dear Examiner Ungar:

Enclosed, please find a copy of the second Brief on Appeal with Exhibits 1-17, filed on April 9, 2002 in triplicate, as indicated on the attached copy of the stamped postcard.

Please advise if you need any additional copies and we will send them to you.

Very truly yours,

Paul Fehlner/MAK

Paul F. Fehlner, Ph.D.

PFF:mak
Enclosures

cc: Anna Lövqvist, Ph.D.

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PATENT TRADEMARK OFFICE

Docket No: 1225/0C674

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: David BERD

Serial No.: 08/203,004 Art Unit: 1642

Confirmation No.: 2699

Filed: February 28, 1994 Examiner: Susan UNGAR

For: COMPOSITION AND METHOD OF USING TUMOR CELLS

BRIEF ON APPEAL

April 8, 2002

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

Sir:

This second Brief on Appeal (submitted in triplicate) is submitted in response to the Examiner's Communication mailed March 12, 2002. A first Brief on Appeal was filed on December 28, 2001 together with a Petition for a five-month extension of time and the appropriate fee, following the Notice of Appeal filed

May 29, 2001 and the response to the Final Office Action mailed on November 29, 2000. It is believed that no additional fees are required for these submissions. However, should it be determined that additional fees are required or that any refund is due in connection with this application, the Commissioner is hereby authorized to charge the required fee(s) and/or credit the refund(s) due to Deposit Account No. 04-0100.

1. The Real Party in Interest

Thomas Jefferson University (TJU), Philadelphia, Pennsylvania, is the assignee of this application. Avax Technologies, Inc., of Overland Park, Kansas (Avax), has an exclusive license from TJU. Accordingly, Avax is a real party in interest.

2. Related Appeals and Interferences

There are no related appeals or interferences.

3. Status of Claims

Claims 43, 44, 47, 49-62, 64-72, and 74-77 are pending and the final rejection of all of these claims is the subject of this Brief. A copy of the pending claims is attached as an Appendix immediately following this Brief.

4. Status of Amendments

Appellant filed an amendment May 29, 2001 in response to the Final Office Action dated November 29, 2000. The Examiner entered this amendment pursuant to the Advisory Action dated July 5, 2001 (copy attached as Exhibit 1).

5. Summary of the Invention

The present invention concerns a composition comprising human tumor cells (other than melanoma cells) conjugated with a hapten.[1] (Specification, page 8, lines 4-6; page 15, lines 4-9). Such haptenized tumor cells have been surprisingly and unexpectedly discovered to form an effective immunogenic component in a vaccine composition for immunotherapy of cancer of the type from which the cells were

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[1] A hapten is a small molecule that, when conjugated to a carrier, can elicit a specific immune response. Preferred haptens include the highly reactive dinitrophenyl and trinitrophenyl groups. (Specification, page 15, lines 4-9).

derived. (Specification, page 11, line 26 to page 12, line 1; page 17, lines 6-16). The haptenized tumor cells are prepared from tumor cells retrieved from the patient receiving treatment (*i.e.*, they are "autologous"), and are haptenized and rendered incapable of growing in the body of a human before injection therein. (Specification, page 12, lines 1-5 and 19-22; page 15, line 26 to page 16, line 13).

In another aspect, the invention provides a method for treating a malignant tumor (other than melanoma) in a human patient by co-administering a composition comprising haptenized autologous human tumor cells, of the same tumor type as the tumor in the patient, with an adjuvant. (Specification, page 8, lines 6-10; page 11, line 27 to page 12, line 5; page 14, lines 3-20; page 15, line 20). The composition elicits at least one of the following responses upon administration to the patient with the adjuvant: an inflammatory immune response against the tumor of the patient; a delayed-type hypersensitivity response against the tumor of the patient; and activated T lymphocytes that infiltrate the tumor of the patient. (Specification, page 17, lines 6-13; page 18, lines 6-13).

In a further aspect, the invention provides a method for treating a malignant tumor in a human patient by co-administering a composition comprising haptenized autologous human tumor cells of the same tumor type as the tumor in the patient, along with an adjuvant, at least six times. (Specification, page 14, lines 7-18 and 25-26; page 22, lines 22-26) . In still a further embodiment, the patient is given

a dose of cyclophosphamide prior to the first administration of the composition. (Specification, page 21, lines 23-25).

The composition of the invention represents an advance over prior experiments involving haptenization of tumor and other cells for testing in animal models. (Specification, page 17, lines 3-16). Prior art experiments suggested that haptenization results in hapten-specific immunity and, in certain experimental animal models, could elicit immunity to unmodified cells as well. (Specification, page 2, line 25 to page 4, line 28). However, the model systems left unresolved whether a human therapy was possible, as hapten-specific immunity would not be useful against metastasized tumor cells or tumor cells remaining after tumor resection, because the residual tumor cells in a patient do not bear hapten.

The invention addresses a need in the art for an effective immunotherapy for tumors, especially non-melanoma tumors. (Specification, page 11, lines 21-25). Administering haptenized tumor cells unexpectedly increases the effectiveness of the resulting tumor-specific immune response, especially with six or more immunizations, resulting in a more effective immunotherapy. (Specification, page 22, lines 25-26). Most importantly, the inventor has discovered that the protective immunity is not hapten-specific, which the prior art suggested would be the case. (Specification, page 17, lines 3-16; page 23, lines 9-11).

6. Issues

The only remaining issues in this application concern obviousness of the claims over various combinations of references as set forth below.[2]

a. Rejection over Murphy in view of '704 Patent, '843 Patent, '183 Patent, or '001 Patent; Berd 1989; and Geczy

Claims 47, 65-72 and74-77 stand rejected as allegedly being obvious (see paragraph No. 5 of the Final Office Action [Exhibit 2], referencing Paper No. 41 [Exhibit 3], Section 5, pages 2-3 and Paper No. 36 [Exhibit 4], Section 10, pages 8-12) over Murphy et al. (Lab Invest 1990;62:70A; hereinafter "Murphy" [Exhibit 5]), in view of U.S. Patent No. 5,702,704 (hereinafter "'704 patent" [Exhibit 6]), U.S. Patent No. 5,626,843 (hereinafter "'843 patent" [Exhibit 7]), U.S. Patent No. 5,008,183 (hereinafter "'183 patent" [Exhibit 8]), or U.S. Patent No. 4,232,001 (hereinafter "'001 patent" [Exhibit 9]); Berd *et al.*, (Proc AACR 1989:30:382;

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[2] The Examiner withdrew the rejection of claims 43, 49-51, and 54-55 for allegedly not being enabled by the disclosure in the Advisory Action (Exhibit 1) in view of the amendment of claim 43 to recite that the composition of the invention elicits, when administered together with an adjuvant, an immune response. In a previous Office Action, the Examiner stated that the specification enables a method for treating a malignant tumor in a human patient comprising administering the composition of claim 43 (*i.e.*, haptenized autologous non-melanoma tumor cells) and BCG (Office Action dated April 28, 1999 [Exhibit 4; Paper No. 36], paragraph No. 6). The specification supports this recitation, *e.g.*, Examples 2 and 3 report eliciting a striking inflammatory response when the composition of the invention was administered together with the adjuvant BCG. Claims 49-51 and 54-55 depend from claim 43.

hereinafter "Berd 1989" [Exhibit 10]), and Geczy et al. (J Immunol. 1970;19:189-203, hereinafter "Geczy" [Exhibit 11]).

b. Rejection over Berd 1989 in view of '704 Patent, '843 Patent, '183 Patent, or '001 Patent; and Geczy

Claims 47, 65-72, and 74-77 stand rejected as allegedly being obvious over Berd 1989 in view of the 704 Patent, the '843 Patent, the '183 Patent, or the '001 Patent, and further in view of Geczy (see paragraph 6 of the Final Office Action [Exhibit 2], referencing Paper No. 41 [Exhibit 3], Section 6, page 4 and Paper No. 36 [Exhibit 4], Section 11, pages 12-15).

c. Rejection over Berd 1989 in view of 704 Patent, '843 Patent, '183 Patent, or '001 Patent; Geczy, and Wiseman

Claims 43, 44, 47, 49-62, 64-72, and 74-77 stand rejected as allegedly unpatentable over Berd '89 in view of the 704 Patent, the '843 Patent, the '183 Patent, or the '001 Patent; and Geczy, in further view of Wiseman et al. (West J Med 1989;151:283-288, hereinafter "Wiseman" [Exhibit 12]) (see paragraph 7 of the Final Office Action [Exhibit 2], referencing Paper No. 41 [Exhibit 3], Section 7, pages 4-5 and Paper No. 36 [Exhibit 4], Section 12, pages 15-18).

d. Rejection over Berd 1989 in view of '704 Patent, '843 Patent, '183 Patent, or '001 Patent; Geczy, and Berd 1983

Claims 43, 44, 47, 49-62, 64-72, and 74-77 stand rejected as allegedly being unpatentable over Berd '89 in view of the '704 Patent, the '843 Patent, the '183 Patent, or the '001 Patent; and Geczy, in further view of Berd et al. (PASCO 1983;2:56, hereinafter "Berd 1983 [Exhibit 13]) (Final Office Action [Exhibit 2], paragraph No. 8, referencing Paper No. 41 [Exhibit 3], Section 8, page 6 and Paper No. 36 [Exhibit 4], Section 13, pages 18-21).

e. Rejection over Berd 1989 in view of '704 Patent, '843 Patent, '183 Patent, or '001 Patent; Geczy, Sanda and Moody

Claims 43, 44, 47, 49-62, 64-72, and 74-77 remain rejected as allegedly being unpatentable over Berd 1989 in view of the '704 Patent, the '843 Patent, the '183 Patent, or the '001 Patent; and Geczy, in further view of Sanda et al. (J Cellular Biochem 1993;suppl.17D:120, hereinafter "Sanda" [Exhibit 14]) and Moody et al. (J Urol 1991;145:293A, hereinafter "Moody" [Exhibit 15]) (Final Office Action [Exhibit 2], paragraph No. 9, referencing Paper No. 41 [Exhibit 3], Section 9, page 7 and Paper No. 36 [Exhibit 4], Section 14, pages 21-25).

7. Grouping of Claims

The claims do not stand or fall together. In this section, three groups of claims, designated Group A-C, with distinct patentability considerations are identified. In section 8 below, Appellant describes why the claims in Groups A-C are believed to be separately patentable.

Group A: Claims 43 and 49-55. These claims are directed to compositions that have distinct features and patentability considerations. Within this group, claim 49 is directed to a Markush group of tumors that has distinct patentability considerations relative to the genus of tumors. In addition, claim 51 is directed to a specifically recited hapten.

Group B: Claims 44, 56-62, 64, and 76. These claims are directed to a method for treating a malignant tumor in a human, which has distinct patentability considerations relative to the composition claims. Within this group, claims 56 and 57 are directed to a Markush group of tumors that has distinct patentability considerations relative to the genus of tumors. In addition, claim 59 is directed to a specifically recited hapten.

Group C: Claims 47, 65-72, 74, 75, and 77. These claims are directed to a method for treating a malignant tumor in a human, which has distinct patentability considerations relative to the composition claims and to the other method of treatment claims because these claims (i) do not exclude treatment of melanoma tumors and (ii) require at least six administrations of the immunotherapy vaccine. The Examiner has rejected these claims for different reasons than the other claims, which further establishes that these claims stand or fall separately from the other claims.

Within this group, claims 65 and 66 are directed to a Markush group of tumors that has distinct patentability considerations relative to the genus of tumors. In addition, claim 68 is directed to a specifically recited hapten. Finally, claim 70 recites a specific regiment for administration of cyclophosphamide (CY).

8. Argument

The first part of this section summarizes the rejections as they pertain to each claim group, and some general considerations that are important to all issues raised in the Final Office Action. The second part of this section discusses each issue (*i.e.*, obviousness rejection) separately, explaining why each claim group involved in the same rejection, where applicable, is believed to be separately patentable.

Table 1. Rejected Claims and Cited References

No.	Reference Cited in Rejection	Rejected Claims		
		Group A	Group B	Group C
a	Murphy in view of Antibody Patents,* Berd 1989, and Geczy	-	76	47, 65-72, 74-75, 77
b	Berd 1989 in view of Antibody Patents* and Geczy	-	76	47, 65-72, 74-75, 77
c	Berd 1989 in view of Antibody Patents,* Geczy, and Wiseman	43, 49-55	44, 56-62, 64, 76	47, 65-72, 74-75, 77
d	Berd 1989 in view of Antibody Patents,* Geczy, and Berd 1983	43, 49-55	44, 56-62, 64, 76	47, 65-72, 74-75, 77
e	Berd 1989 in view of Antibody Patents,* Geczy, Sanda and Moody	43, 49-55	44, 56-62, 64, 76	47, 65-72, 74-75, 77

* "Antibody Patents" = '704, '843, '183, and '001 Patents

The relevant test for obviousness requires three basic factual inquiries: the scope and content of the prior art are to be determined; the differences between the prior art and the claims at issue are to be ascertained; and the level of ordinary skill in the art resolved. *Graham v. Deere*, 383 U.S. 1, 17 (1966); *Ruiz v. A.B. Chance Co.*, 57 USPQ2d 1161, 1165 (Fed. Cir. 2000). The relevant inquiry involves three steps. First, there must be some suggestion or motivation to modify what is taught in a reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference or combination of references must teach all of the claim limitations. Both the motivation and the reasonable expectation of success must be found in the prior art, not in appellant's disclosure.

See, MPEP § 2143, *citing In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

As explained in detail below, the Final Office Action fails to establish a *prima facie* case for obviousness under these requirements because there is no motivation to combine the references, and, even when forcibly combined, they do not provide a reasonable expectation of success.

In all of the rejections, the Examiner relies on the Berd 1989 abstract as an allegedly successful example of treatment of melanoma by administration of DNP-conjugated autologous melanoma cells in connection with BCG and a preceding dose of cyclophosphamide. The Examiner also contends that it would have been expected that the autologous irradiated melanoma, lung, colon, kidney, and colon cancer cells of Wiseman ([Exhibit 11] discussed below) would be successfully substituted for the melanoma cells of Berd 1989 to treat other cancer types (Final Office Action [Exhibit 2], bridging paragraph between pp. 5 and 6). However, both of these conclusions depend on according more weight to the Abstract than one of ordinary skill at the time of the invention would have given it.

To factually determine what a reference teaches one of ordinary skill in the art in implementing the Graham standard, the courts have relied upon affidavit evidence either by experts or those of ordinary skill in the art. *See In re Carroll* 202 USPQ 571 (CCPA 1979); *In re Piasecki*, 223 USPQ 785, 789 (Fed. Cir. 1984); *In re Oelrich*, 198 USPQ 210 (CCPA 1978). Furthermore, affidavits of those skilled in the

art have been held to constitute factual evidence of the level of skill in the art. *E.g., In re Piasecki*, 223 USPQ at 789; *In re Oelrich*, 198 USPQ 210, 214-15. Such affidavits constitute competent evidence that cannot be ignored. *See e.g., Ashland Oil, Inc. v. Delta Resins & Refractories, Inc.*, 227 USPQ 657, 674-75 (Fed. Cir. 1985).

Appellant has therefore addressed the teachings of this reference through the lense of one of ordinary skill in the art, Dr. Donald Braun, who attended an interview with the Examiner and her supervisor on January 5, 2000.[3] As set forth by the Braun Declaration [Exhibit 16] accompanying the response to the Final Office Action[4], Berd 1989 does not describe a successful immunotherapy for melanoma (Braun Declaration, paragraph 7). On the contrary, it represents a preliminary result that raises more questions and ambiguities than it answers. Early animal work on tumor immunotherapy could not establish whether similar approaches could work in

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[3] Dr. Donald Braun has a long career in the field of immunological oncology, as evidence by his *curriculum vitae*, attached as Exhibit A to the Braun Declaration [Exhibit 16]. There can be no doubt as to his qualifications as one of at least ordinary skill in the art.

[4] The Braun Declaration memorializes comments made by Dr. Braun during the personal interview with the Examiner and her Supervisor on January 5, 2001, at which this application and a number of related applications by the same inventor were discussed. The points made therein reinforce scientific and factual argument distinguishing the prior art of record already made by applications. The Examiner agreed at the interview that Dr. Braun's Declaration would substantiate these points. However, for reasons unknown to the Appellant, the Examiner stated in the Advisory Action (Exhibit 1) that she had not considered the Braun Declaration "... because Applicant has not shown good and sufficient reasons why it was not earlier presented..." (Advisory Action [Exhibit 1], page 2). It had seemed self-evident that presentation of this Declaration could not have preceded the clarification of issues achieved at the interveiw.

humans (Braun Declaration, paragraph 8). The Abstract fails to provide a definitive protocol that would permit one to repeat the work, determine whether this approach elicited an immune response to unmodified cells, or establish that it achieved any clinical benefit (Braun Declaration, ¶¶ 9, 10, 11).

Thus, since the primary reference, Berd 1989, fails to provide any expectation of success, *i.e.*, clinical benefit, using the haptenized tumor cell approach in melanoma patients, this reference is completely irrelevant in providing any expectation of success for such an approach in other types of cancer. Since no other reference cited by the Examiner makes up for this fundamental flaw, nor the combination of them (see below), obviousness does not obtain.

With these considerations in mind, we turn to each issue, and the specific grounds for rejection.

a. The Rejection of Claims 47, 65-72, and 74-77 over Murphy in view of '704 Patent, '843 Patent, '183 Patent, or '001 Patent; Berd 1989; and Geczy

The rejected claims are all part of Group C, except for claim 76, which is part of Group B. Claim 76 is directed to a method for treating a non-melanoma malignant tumor in a human patient by co-administering a composition comprising haptenized autologous human tumor cells of the same tumor type as the tumor in the patient, with an adjuvant. The broadest claim of Group C, claim 47, is directed to a method for treating a malignant tumor in a human patient by co-administering a

composition comprising haptenized autologous human tumor cells of the same tumor type as the tumor in the patient, along with an adjuvant, at least six times. As disclosed in Examples 3, 4, and 6 of the instant application, administration of the immunotherapeutic vaccine comprising haptenized tumor cells on at least six, and in most cases eight, occasions resulted in actual treatment of tumors, with statistically significant greater cancer-free survival compared to controls (who received non-haptenized vaccine) at two years. (Specification, page 29, lines 22-25). The difference was highly significant. (*Id.*, page 30, lines 26-27; page 41, line 24 to page 42, line 8).

i. ***The Examiner's Reasoning***

The Examiner states that Murphy teaches a method for treating melanoma comprising sensitizing with DNCB, administering a therapeutically effective amount of cyclophosphamide (CY), and administering a therapeutically effective amount of autologous, irradiated DNP-conjugated melanoma cells mixed with BCG adjuvant. The Examiner notes that Murphy does not teach administration of at least six vaccine doses at spaced intervals, a specific dose of CY (300 mg/M^2), prior sensitization with 1-fluoro-2,4-dinitrobenzene, or eliciting certain specified immune responses against the tumor. The Examiner cites the '704, '843, '183, and '001 Patents for teaching administration of at least six doses of antigen; Berd 1989 for teaching a successful

method of treating melanoma with the specified dosage of CY using DNP-conjugated melanoma cells, and Geczy for teaching halogenated dinitrobenzenes for eliciting delayed type hypersensitivity. (Paper 36 [Exhibit 4], pages 9-10). The thrust of this rejection, then, is that it would have been obvious from the combined teachings of Murphy; the '704, '843, '183, or '001 Patents; and Berd 1989 to administer at least six doses of a haptentized tumor cell vaccine, and that various haptenization reagents can be used as described by Geczy.

Appellant have previously argued that the references cannot be combined as suggested by the Examiner without employing impermissible hindsight from the disclosure of the invention. The Examiner contends that "[s]ome degree of hindsight is permissible in making rejections under 35 U.S.C. 103 since it must be recognized that any judgement on obviousness is in a sense necessarily a reconstruction based upon hindsight reasoning." (Final Office Action [Exhibit 2], page 3).

ii. ***Appellant's arguments***

The teachings of ***Berd 1989*** has been discussed above and in the Braun Declaration [Exhibit 16], noting in particular the lack of guidance and expectation of success of this reference.

As noted in Appellant's amendment filed September 22, 2000 on page 14, paragraph 3.d, ***Murphy's*** teachings are cumulative to those of Berd 1989 in all aspects relied upon by the Examiner, thereby suffering from the same lack of guidance and expectation of success for the haptenized-tumor-cell approach in melanoma, and, of course, even more so in the case of non-melanoma tumors.

The teachings and deficiencies of ***Geczy*** have been discussed in Appellant's amendment filed September 22, 2000, on pages 11-12, paragraphs 3.b.iii. Geczy proposes that direct haptenization of lymphocytes is necessary for lymphocyte transformation, thereby primarily relating to anti-hapten responses. Geczy does not pertain to cancer therapy. In addition, Geczy's anti-hapten responses would not be useful for tumor treatment, since they would attack the haptenized tumor cell vaccine itself instead of residual tumor cells. Thus, to the extent that the teachings of Geczy relate to those of Berd 1989 and/or Murphy, they diverge and teach away from using haptens to elicit a protective immune response against unmodified tumor cells.

With respect to the ***'704, '843, '183, and '001*** Patents, their teachings and deficiencies were also discussed in the amendment dated September 22, 2000, pages 10-11, paragraph 3.b.ii. Since the teachings of these patents are substantially overlapping, they are hereinafter collectively referred to as the "Antibody Patents."

The immunization schedules proposed in the Antibody Patents result in the generation of antibodies against the antigens. Such antibodies can be useful as diagnostic reagents, but since the subjects do not develop protective immunity to the immunogen(and are not intended to do so), the Antibody Patents are irrelevant for immunotherapy treatment regimes. In fact, the Antibody Patents fail to suggest, and indeed teach away from, generating an immune response to a carrier, since the goal of the references is to elicit an immune response to a "carried" substance, *i.e.*, the antigen, and not a carrier.[5] This is directly analogous to eliciting an anti-hapten antibody response against a hapten "carried" by a tumor cell. As pointed out above, such a result would be contrary to the invention, since an anti-hapten immune response would not affect residual tumor cells remaining after resection or metastasis. Thus, the Antibody Patents, as well as Geczy, teach away from a key point of the invention; the elicitation of an immune response towards unmodified tumor cells (the "carriers").

Finally, the Antibody Patents do not teach or suggest at least six administrations or immunizations for the treatment of cancer. No objective teaching thus exists in these patents that would suggest to or motivate one of skill in the art to administer antigens or antibodies to humans at least six times at spaced intervals in order to treat cancer.

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[5] See, *e.g.*, the '704 patent, column 13, line 38 to column 14, line 9.

In conclusion, there is no motivation to combine references that relate to immunotherapy of cancer and anti-tumor responses (Murphy, Berd 1989) with references which impliedly or explicitly teaches anti-hapten responses and do not even relate to cancer treatment (Geczy, Antibody Patents). Even when forcibly combined, however, there are no teachings about treatment of non-melanoma tumors, or about ***successful*** treatment of ***any*** tumor type, in this combination of references.

iii. *Separate Patentability Issues*

Claim 76 (Group B) cannot be obvious over the cited combination of references because (1) the references cannot be combined (see preceding section), and (2) the combination does not teach treatment of ***non-melanoma*** tumors, an explicit feature of the claim.

The claims of Group C cannot be obvious over the cited combination of references because (1) the references cannot be combined (see preceding section), and (2) even if forcibly combined, there would be no reasonable expectation that six or more administrations of vaccine would yield a more successful tumor treatment as demonstrated in the Examples. If anything, the combined teachings of the references leads one of ordinary skill to predict that six or more administrations of a haptenized tumor cell vaccine would yield a stronger anti-hapten antibody response, e.g., as shown by the Antibody Patents. Such an outcome leads away from the Group C

claims in particular, as these claims all require at least six administrations of vaccine.

Thus, there are different reasons for the non-obviousness of claim 76 and the claims of Group C over the cited combination of references. In addition, claim 76 depends from claim 44, which was not included by the Examiner in this rejection. Accordingly, claim 76 and Group C are separately patentable.

iv. *Errors in Rejection*

The Examiner has made a number of legal errors to arrive at a conclusion of obviousness based on the combined teachings of these references, primarily by failing to properly articulate the Graham factors. For example, Examiner did not properly consider the scope and content of the prior art, and the differences between the prior art and the claimed invention. *Ruiz*, 57 USPQ2d at 1167. The Examiner further failed to establish the level of ordinary skill in the art, *Id.* at 1168, which Appellant has established through the Braun Declaration as well as through the references cited by the Examiner.

With respect to considering the scope and content of the prior art and the differences between the prior art and the claimed invention, the Examiner failed to articulate "... a reason, suggestion, or motivation in the prior art or elsewhere that would have led one of ordinary skill in the art to combine the references." *Id.* at 1167, *citing In re Rouffet*, 47 USPQ2d 1453, 1459 (Fed. Cir. 1998) and *In re Dembiczak*,

50 USPQ2d 1614, 1617 (Fed. Cir. 1999). The Federal Circuit provides explicit guidance in *Ruiz* on the factual findings to make in determining a reason, suggestion, or motivation to combine:

> The reason, suggestion, or motivation to combine may be found explicitly or implicitly: 1) in the prior art references themselves; 2) in the knowledge of those of ordinary skill in the art that certain references, or disclosures in those references, are of special interest or importance in the field; or 3) from the nature of the problem to be solved, "leading inventors to look at references relating to possible solutions to that problem."

Id. (citations omitted). The Examiner merely alludes to a "conventional immunization scheduled" (Final Office Action [Exhibit 2], page 4; Paper 36 [Exhibit 4], page 11) without providing any basis for linking a conventional immunization schedule for eliciting diagnostic antibodies to an immunotherapy regimen.

The error here arises from the Examiner falling "into the hindsight trap." *In re Kotzab*, 55 USPQ2d 1313, 1318 (Fed. Cir. 2000). As the Court stated in *Kotzab*, "... to establish obviousness based on a combination of the elements disclosed in the prior art, there must be some motivation, suggestion or teaching of the desirability of making the specific combination that was made by the applicant." *Id.* at 1316, *citing In re Dance*, 48 USPQ2d 1635, 1637 (Fed. Cir. 1998); *In re Gordon*, 211 USPQ 1125, 1127 (Fed. Cir. 1984). The Antibody Patents teach methods to elicit antibodies to the immunizing agent, *e.g.*, a hapten-like compound such as an advanced glycosylation end-product (*see* the '704 patent, Exhibit 6). Geczy shows that haptenization results in hapten-specific immunity. Neither Murphy nor Berd 1989

suggest at least six administrations of the immunotherapeutic vaccine, much less the advantages of doing so disclosed in the Examples of the instant application. Thus, the Examiner has "... found prior art statements that in the abstract appeared to suggest the claimed limitation...", *Id.* at 1318, but which, in fact, lack any motivation to modify the teachings of Murphy or Berd 1989 to include that limitation.

With respect to the level of skill in the art, which the Examiner relies on in making this rejection (Final Office Action [Exhibit 2], page 4), Appellant respectfully submits that the Braun Declaration and the explicit teachings of the references show that the level of skill in the art does not supply the missing teaching here. *See A-Site Corp. v. VSI*, 50 USPQ2d 1161, 1171 (Fed. Cir. 1999). "[T]he level of skill in the art is a prism or lens through with a judge or jury views the prior art and the claimed invention. This reference point prevents these deciders from using their own insight or, worse yet, hindsight, to gauge obviousness." *Id.* The Examiner has not established that the level of skill in the art is such that it would lead the skilled artisan to modify the teachings of Murphy or Berd 1989 as set forth in the claims. In particular, there is no incentive to administer vaccine six times (claims of Group C) or to treat non-melanoma tumors (claims of Group B). To rely on "conventional immunization schedules" is therefore in error.

The Examiner cited two cases to support her analysis of obviousness. In particular, the Examiner points out that "[t]he test for obviousness...is what the combined teachings of the references would have suggested to those of ordinary skill

in the art." *In re Keller*, 208 USPQ 871, 881 (CCPA 1981) (Citations omitted). Appellant agrees, but points out that the Examiner must consider the references for all that they teach; it is impermissible to consider a reference in less than its entirety, or to disregard disclosures that diverge and teach away. *W.L. Gore & Assoc., Inc. v. Garlock, Inc.*, 220 USPQ 303 (Fed. Cir. 1993), *cert. denied* 469 U.S. 851 (1984). Such teaching away, which is the effect of the Antibody Patents and Geczy reference, defeats obviousness. *See Winner Int'l Royalty Corp. v. Wang*, 53 USPQ2d 1580, 1587 (Fed. Cir. 2000). Moreover, the prior art, and not the disclosure in the application, must both suggest the invention and provide a reasonable expectation of success in achieving it. *In re Vaeck*, 20 USPQ 2d 1438 (Fed. Cir. 1991).

Correctly applying the foregoing legal principles, the references neither (1) suggest the invention, nor (2) provide a reasonable expectation of success. With respect to the claims of Group C, Berd 1989 and, by extension, Murphy, are inadequate to suggest modifying the immunization strategy to require at least six administrations of an immunotherapeutic vaccine. With respect to claim 76, the Examiner has not alleged any basis for rejecting a claim directed to treatment of tumors other than melanoma. Taken together, which as the Examiner has pointed out is how the references must be considered, the references do not render the claimed invention obvious. The Examiner's failure to properly consider the *Graham* factors, and the admitted use of hindsight to establish obviousness, represent error and should be reversed.

b. The rejection of claims 47, 65-72 and 74-77 over Berd 1989, the Antibody Patents, and Geczy

This rejection also pertains to the claims of Group C, with the exception of claim 76 (Group B). The Examiner maintained this rejection for the same reasons described in the above rejection. For the reasons set forth above, the combination of Berd 1989, the Antibody Patents, and Geczy, fails to render the instant invention obvious. Berd 1989, like Murphy, provides little to no information about method of treatment and therapeutic, which the Braun Declaration makes abundantly clear. In short, one of ordinary skill in the art would not have had any motivation to modify the teachings of Berd 1989 to require immunization with the haptenized tumor cell vaccine at least six times, and none of the cited references teaches treatment of non-melanoma tumors. The same reasons for separate patentability applies here as in the previous rejection. The Examiner has erroneously failed to establish the *Graham* factors sufficient to render the invention obvious, and instead has substituted hindsight to sustain this rejection, both of which constitute error. *See Ruiz*, 57 USPQ2d at 1167-68. Thus, this rejection is in error and should be reversed.

c. The rejection of claims 43, 44, 47, 49-62, 64-72, and 74-77 over Berd 1989, the Antibody Patents, and Geczy in view of Wiseman

This rejection pertains to claims in all of Groups A, B, and C. The broadest claim in Group A, claim 43, is directed to a composition comprising human

tumor cells (other than melanoma cells) conjugated with a hapten. The tumor cells to be haptenized are obtained from the patient receiving treatment (*i.e.*, they are "autologous"), and are rendered incapable of growing in the body of a human before injection therein.

The broadest claim in Group B, claim 44, is directed to a method for treating a malignant tumor (other than melanoma) in a human patient by co-administering a composition comprising haptenized autologous human tumor cells of the same tumor type as the tumor in the patient, along with an adjuvant. The composition elicits at least one of the following upon administration to the patient with the adjuvant: an inflammatory immune response against the tumor of the patient; a delayed-type hypersensitivity response against the tumor of the patient; and activated T lymphocytes that infiltrate the tumor of the patient.

The broadest claim of Group C, claim 47, has been discussed above.

i. ***The Examiner's Reasoning***

All references and the Examiner's reasons for combining them have been discussed above, except for Wiseman. The Examiner contends that "Wiseman clearly showed that autologous irradiated melanoma, lung, colon, and kidney cancer were successfully used for successful immunological treatment of those cancers and it would have been expected that these cell types, already known in the art to be useful

as immunogenic cancer treatments would be successfully substituted for the melanoma cells of Berd [1989] in order to treat the other cancer types." (Final Office Action [Exhibit 2], paragraph bridging pages 5 and 6).

ii. ***Appellant's arguments***

The Braun Declaration addresses the teachings and deficiencies of the primary reference, ***Berd 1989***, as discussed above. Briefly, Berd 1989 lacks teachings with respect to any clinically significant tumor regression being observed, as well as the numbers and route of administration. (Braun Declaration [Exhibit 16], ¶¶9 and 11). Thus, one of ordinary skill in the art would have presumed that Berd 1989's haptenized tumor cells and BCG had been injected intra-tumorally, and that the BCG was thereby responsible for the observed, clinically non-significant, tumor responses (Braun Declaration [Exhibit 16],¶11). Accordingly, Berd 1989 suffers from a lack of expectation of success for the haptenized-tumor-cell approach in melanoma, and even more so in the case of non-melanoma tumors.

As discussed in the amendment dated September 22, 2000 (p. 17, 1st full paragraph), ***Wiseman*** teaches an alternative form of immunotherapy that depends on the route of administration: intralymphatic immunization. This alternative, which

Wiseman indeed reports favorably, in no way suggests a deficiency or problem that would lead one of ordinary skill in the art to seek an alternative immunization strategy.

On the contrary, Wiseman diverts the skilled artisan away from the claimed invention, thus precluding combining this reference in making the rejection. One of ordinary skill in the art would, when provided with the Wiseman reference on one hand and Berd 1989 on the other, be inclined to pursue the Wiseman approach: intralymphatic immunization with unmodified tumor cells, since this approach was successful and avoided the problems with the haptenization approach in Berd 1989. Finally, Geczy and the Antibody Patents lead away from any notion that haptenized tumor cells could yield an anti-tumor cell response, because they suggest an anti-hapten response, and, in addition, do not relate to cancer treatment. Such teaching away, which is the effect of the Antibody Patents and Geczy reference, defeats obviousness. *See Winner Int'l Royalty*, 53 USPQ2d at 1587.

As discussed above, nothing in Berd 1989 suggests that haptenization of tumor cells provides an effective therapeutic response (as established by the Braun Declaration), much less a more effective response than other immunization protocols. However, the data in the present specification clearly supports the unexpected superiority of the haptenized tumor cells and methods of immunotherapy using them.

> The DFS [disease-free survival] and TS [total survival] of [patients treated with haptenized tumor cells] were compared with those of 22 melanoma

> patients with resected nodal metastasises treated previously with unconjugated vaccine, see Example 4. Of 36 patients with stage 3 melanoma (palpable mass in one lymph node region), 22 are disease-free with a median follow-up of 33 months. Kaplan-Meir analysis projects a 3 year DFS and TS of 59% and 71%, respectively. In contrast, the DFS and TS of stage 3 patients treated with unconjugated vaccine was 22% and 27% respectively (p = 0.01, log-rank test). Of 11 stage 4 patients (palpable mass in two lymph node regions), 5 are NED (no evidence of disease) with a median follow-up of 41 months.

(Specification, page 41, line 24 to page 42, line 8). These data demonstrate the superiority of the claimed invention, particularly the claimed methods of treatment, relative to Wiseman's approach.

These advantages, however, can only be gleaned from the disclosure of the specification, and are not available from the combined teachings of the references. Advantages flowing directly from the invention are one consideration that may be relevant to a determination of obviousness. *Mosinee Paper Corp. v. James River Corp. of Virginia*, 22 U.S.P.Q.2d 1657, 1660, *aff'd. mem.* 980 F.2d 743 (Fed. Cir. 1992) (citing *Pre-Emption Devices, Inc. v. Minnesota Mining & Mfg. Co.*, 221 USPQ 841 (Fed. Cir. 1984)). "After all, those advantages are the foundation of that 'commercial success' which may be evidence of nonobviousness." *Pre-Emption, supra*, at 844 (*citing Graham*, 383 U.S. at 17). Thus, the showing of significant advantages of the presently claimed compositions and methods, particularly as related in Example 6 (quoted above), demonstrates non-obviousness of the invention.

Even if Berd 1989 ***had*** taught an effective immunotherapy of melanoma using haptenized, autologous melanoma cells, such a teaching would not form a sufficient basis for combination with Wiseman to achieve the claimed invention. As applicants have previously pointed out, it is not expected that "vaccines using other types of tumor cells, shown to effectively treat cancer, would behave in a mechanistically similar manner to the melanoma vaccine described in Berd et al." (See Paper No. 41 [Exhibit 3], page 5, lines 12-14). In the PTO-1449 form filed by Applicant on December 1, 1998, Applicant brought the Examiner's attention to Hanna et al. (U.S. Patent No. 5,484,596, hereinafter "Hanna"). Hanna teaches a method for the treatment of human colon cancer that involves the use of a vaccine which is made from irradiated human tumor cells. The Examiner was requested to note that the Hanna vaccine strategy appears to be effective only for treating colon cancer. A publication reporting on a clinical trial of the "Hanna" vaccine conceded that the vaccine was not effective for rectal cancer (Hoover et al., J. Clin. Oncology 11: 390-399, 1993; copy attached to the Amendment filed September 22, 2000 as Exhibit 1 [Exhibit 17], "Hoover"). Hoover states that ". . . no benefits were seen in patients with rectal cancer who received [active specific immunotherapy with an autologous tumor cell-BCG vaccine]" (see Abstract; see also page 399, first column). Hence, even though the Hanna vaccine was reportedly successful in treating colon cancer, it failed to provide any benefits to patients with rectal cancer, a tumor type closely related to colon cancer. Accordingly, even had Berd 1989 successfully treated

melanoma patients with his haptenized tumor cell vaccine, and not only provided preliminary and essentially anecdotal results relating to DTH-responses, it could not have been reasonably expected that a similar vaccine would be equally effective in the treatment of related tumors, much less tumors of completely unrelated origin.

It is clear that upon careful examination, the references cannot be combined as the Examiner has suggested. Thus, here the Examiner's citations appear on the surface to suggest the claimed invention, but, upon further review, can only be combined as the Examiner proposes with knowledge of the Applicant's invention. *In re Kotzab*, 55 USPQ2d at 1318. Such an analysis is, of course, improper. For the foregoing reasons, this obviousness rejection is in error and should be reversed.

iii. ***Separate Patentability Issues***

The product claims of Group A cannot be obvious over the suggested combination of references, since the references cannot be combined to teach a haptenized non-melanoma tumor cell vaccine which elicits an anti-tumor response. Berd 1989 merely teaches a melanoma cell vaccine for immunotherapy, and Hanna and Hoover showed that an immunotherapy vaccine successful for one type of tumor would not function as a vaccine for another, closely related, tumor type. Thus, a combination with Geczy and the Antibody Patents and the Wiseman reference would not lead to any suggestion, much less conclusion, that the melanoma vaccine of Berd

1989 would indeed elicit an anti-tumor response if applied to wholly unrelated tumor cells. Thus, there is no incentive from these references to haptenize non-melanoma tumor cells for an immunotherapy vaccine.

The method claims of Group B are not obvious over the suggested combination of references because (1) the references cannot be combined to teach a haptenized non-melanoma tumor cell vaccine which elicits an anti-tumor response; and (2) even when forcibly combined, they do not suggest a reasonable expectation of a successful treatment. The reasons why the references cannot be combined are provided in the preceding paragraph. As for the lack of expectation of success, Berd 1989, using haptenized melanoma cells, does not establish a successful immunotherapy method for melanoma. Geczy and the Antibody Patents suggest that an anti-hapten response would be the one and only result, and Wiseman proposes that intralymphatic injection of unmodified cells is a successful approach. Therefore, this combination of references, while admittedly raising expectations of success for the use of an ***unmodified*** tumor cell vaccine of Wiseman, could not possibly teach a reasonable expectation of success in achieving an anti-tumor response against a non-melanoma tumor using a haptenized vaccine.

Finally, the claims of Group C are not obvious over the (forced) combination of references simply because any administration schedule provided by the Antibody Patents to elicit a response to an antigen, exemplified by the "carrier"-associated glycosylation end-product of the '704 patent, would merely be applicable

for eliciting an anti-hapten response, and not for an anti-tumor response, as previously described. These differences between the references except Wiseman and the Group C claims have been discussed. Wiseman adds nothing about an effective number of administrations for a haptenized tumor cell vaccine. In short, the claims of Group C are patentable and distinct for all the reasons discussed in 8(a) and 8(b), *supra*.

Since the reasons for non-obviousness of the claims in the different claim groups are different, separate patentability considerations apply.

d. The rejection of claims 43, 44, 47, 49-62, 64-72, and 74-77 over Berd 1989, the Antibody Patents, Geczy, and Berd 1983

This rejection pertains to all of Groups A-C. The broadest claims of these claim groups, claims 43, 44, and 47, have been discussed above. The teachings of Berd 1989, the Antibody Patents, and Geczy have also been discussed above.

i. *The Examiner's Reasoning*

The Examiner contends that Berd 1983 teaches treatment of breast cancer patients with autologous vaccine, and that the substitution of the breast cancer cells of Berd 1983 for the melanoma cells of Berd 1989 was *prima facie* obvious. The Examiner also alleges that Appellant previously argued the Berd 1983 reference individually without clearly addressing the combined teachings.

Appellant respectfully disagrees. Appellant chose to, instead of repeating arguments already made in the amendment, discuss the entirety of the teachings of Berd 1983 before adding this reference to the combination of Berd 1989, the Antibody Patents, and Geczy (see amendment dated September 22, 2000, page 18, section 3.g). In doing so, it is clear that Berd 1983 adds nothing to the combination of Berd 1989, the Antibody Patents, and Geczy, which combination is (1) improper and (2) fails to provide any reasonable expectation of success as discussed above.

ii. ***Applicant's Arguments***

Berd 1983 teaches intradermal administration of autologous tumor cells to six cancer patients, five suffering from melanoma and one from breast cancer, and reports DTH responses against tumor cells in three out of the five evaluated patients. Note that Berd 1983 is silent with respect to whether the single breast cancer patient was among the 3 patients (50%) showing a DTH response. Even assuming that the breast cancer patient was among the three, the addition of Berd 1983 to the combination of reference would not provide a reasonable expectation that a haptenized tumor cell vaccine, whether based on melanoma or breast cancer cells, would elicit a clinically significant anti-tumor response.

The Braun Declaration addresses the teachings and deficiencies of ***Berd 1989***, as discussed above. The reference lacks teachings with respect to any clinically significant tumor regression being observed, as well as the numbers and route of administration. (Braun Declaration [Exhibit 16], ¶¶9 and 11). Thus, one of ordinary skill in the art would have presumed that Berd 1989's haptenized tumor cells and BCG had been injected intratumorally, and that the BCG was thereby responsible for the observed, clinically non-significant, tumor responses (Braun Declaration [Exhibit 16],¶11). Accordingly, Berd 1989 suffers from a lack of expectation of success for the haptenized-tumor-cell approach in melanoma, and even more so in the case of non-melanoma tumors.

As noted above, even if Berd 1989 taught an effective immunotherapy of melanoma using haptenized, autologous melanoma cells, such a teaching would not form a sufficient basis for combination with Berd 1983 to achieve the claimed invention. As applicants have previously pointed out, it is not expected that "vaccines using other types of tumor cells, shown to effectively treat cancer, would behave in a mechanistically similar manner to the melanoma vaccine described in Berd et al." (See Paper No. 41 [Exhibit 3], page 5, lines 12-14). As described in section 8.d.ii above, Appellant brought the Examiner's attention to Hanna and to Hoover [Exhibit 17], the latter of which showed that the "Hanna" vaccine, reportedly successful for treating colon cancer, was unsuccessful for treating rectal cancer. Hence, if a vaccine strategy cannot be successfully translated from colon cancer to rectal cancer, a person

of skill could not reasonably expect to translate Berd 1989's melanoma cell vaccine to a successful, wholly unrelated, breast cancer or other non-melanoma vaccine.

iii. ***Separate Patentability Issues***

The teachings of Berd 1983 are the same as those of Wiseman 1983 in the context of the Examiner's rejection discussed under section 8.d herein, and all other references are the same. Therefore, Groups A-C have different patentability considerations for the same reasons as those presented in section 8.d.iii above.

iv. ***Errors in Rejection***

It is clear that upon careful examination, the references cannot be combined as the Examiner has suggested. Thus, even though the Examiner's citations appear on the surface to suggest the claimed invention, upon further review it is discovered that they can only be combined as the Examiner proposes with knowledge of the Applicant's invention. *In re Kotzab*, 55 USPQ2d at 1318. Such an analysis is, of course, improper. For the foregoing reasons, this obviousness rejection is in error and should be reversed.

e. The rejection of claims 43, 44, 47, 49-62, 64-72, and 74-77 over Berd, the Antibody Patents, Geczy, and Sanda and Moody

This rejection pertains to claims in all of Groups A-C. The broadest claims in these claim groups, claims 43, 44, and 47, have been discussed above. Berd, the Antibody Patents, and Geczy, as well as their teachings, are discussed above.

i. *The Examiner's Reasons*

The Examiner has stated that in particular Berd 1989 supplies the motivation to "decorate the tumor cells with hapten." Incorporating the reasoning set forth in the prior two Office Actions, the Examiner states that Moody teaches that lymphokine-transfected prostate cells generate an anti-tumor effect *in vivo*, and that Sanda addresses the feasibility of gene therapy for human prostate cancer. These references appear to be relevant to the Examiner because they suggest methods of anti-prostate cancer therapy.

ii. *Applicant's Arguments*

Sanda teaches a method for transducing human prostate cancer cells with a particular retroviral vector. This approach is wholly unrelated to any immunotherapy method for cancer treatment, and it solves a different problem (tumor cell ablation

through a therapeutic gene) than the claimed invention (tumor cell immunotherapy using a haptenized tumor cell as a vaccine). Thus, there is no logical connection between Sanda and the other references cited in this rejection. *See Ruiz*, 57 USPQ2d at 1168 (evidence that the references solve different problems can preclude a determination of obviousness).

Moody teaches an immunotherapy method based on transfection of rat prostate tumor cells to make them express the lymphokines IL-2 and IL-4. Although reportedly successful, the method disclosed in this reference adopts an altogether different approach than haptenization of tumor cells, and there is no suggestion from Moody to modify his approach by haptenization.[6]

In short, for the reasons discussed above, the forced combination of Berd 1989, the Antibody Patents, and Geczy fail to suggest, much less teach, compositions of haptenized non-melanoma tumor cells, methods of cancer immunotherapy using haptenized non-melanoma tumor cells, or methods of cancer immunotherapy involving a specific regimen of administering haptenized tumor cells at least six times. Sanda and Moody have nothing to do with such immunotherapy; they are in this respect less

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[6] As disclosed in the specification of the instant application, using immunostimulatory molecules in <u>combination</u> with the claimed compositions and methods may be desirable in some instances. (Specification, page 17, lines 17-20). Moody provides one avenue for such a combination. This in no way suggests the claimed invention.

relevant than Wiseman. Accordingly, for the reasons advanced above, this rejection is in error and must be reversed.

iii. *Separate Patentability Considerations*

Sanda and Moody add nothing of relevance for any of Groups A-C, and the distinctions between the claims and all other references considered together are the same. Therefore, Groups A-C have different patentability considerations for the same reasons as those presented in section 8.d.iii above.

iv. *Error's in Rejection*

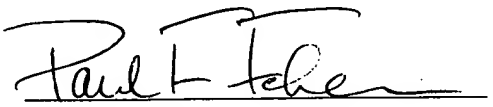
It is clear that upon careful examination, the references cannot be combined as the Examiner has suggested. Thus, even though the Examiner's citations appear on the surface to suggest the claimed invention, upon further review it is discovered that they can only be combined as the Examiner proposes with knowledge of the Applicant's invention. *In re Kotzab*, 55 USPQ2d at 1318. Such an analysis is, of course, improper. For the foregoing reasons, this obviousness rejection is in error and should be reversed.

9. Conclusion

For the forgoing reasons, Appellant submits that the Final Rejection is in error and should be reversed on all grounds. The Examiner has committed error by failing across the board to properly articulate the *Graham* factors. For example, Examiner did not properly consider the scope and content of the prior art, and the differences between the prior art and the claimed invention. *Ruiz*, 57 USPQ2d at 1167. The Examiner further failed to establish the level of ordinary skill in the art, *Id.* at 1168, which Appellant has established through the Braun Declaration as well as through the references. With respect to considering the scope and content of the prior art and the differences between the prior art and the claimed invention, the Examiner failed to articulate "... a reason, suggestion, or motivation in the prior art or elsewhere that would have led one of ordinary skill in the art to combine the references." *Id.* at 1167 (citations omitted). Instead, the Examiner has improperly relied on an arbitrary, non-existent level of skill in the art to fill in the holes in the prior art, *See A-Site Corp.* 50 USPQ2d at 1171, and has consistently fallen into the "hindsight trap". *In re*

Kotzab, 55 USPQ2d at 1318. For these reasons, reversal of all rejections and remand of the application for allowance is believed to be in order and is earnestly solicited.

Respectfully submitted,



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APPENDIX

Pending Claims on Appeal

43. (Amended) A composition comprising human tumor cells that:

(i) are conjugated to a hapten;

(ii) are of the same tumor type as a malignant tumor of a patient for treatment of whom the composition is intended;

(iii) are autologous to said patient; and

(iv) have been rendered incapable of growing in the body of a human upon injection therein;

said composition eliciting, when administered together with an adjuvant, an inflammatory immune response against the tumor of said patient, wherein said tumor is not melanoma.

44. A method for treating a malignant tumor in a human patient comprising co-administering to the patient

(a) a composition comprising a therapeutically effective amount of human tumor cells that:

(i) are conjugated to a hapten;

(ii) are of the same tumor type as a malignant tumor of a patient for treatment of whom the composition is intended;

(iii) are autologous to said patient; and

(iv) have been rendered incapable of growing in the body of a human upon injection therein; and

(b) an adjuvant;

wherein said composition elicits at least one of the following upon administration to said patient with the adjuvant: an inflammatory immune response against the tumor of said patient; a delayed-type hypersensitivity response against the

tumor of said patient, and activated T lymphocytes that infiltrate the tumor of said patient, wherein said malignant tumor is not melanoma.

47. (Amended) A method of treating a malignant tumor in a human patient comprising co-administering to the patient

(a) a composition comprising a therapeutically effective amount of human tumor cells that:

(i) are conjugated to a hapten;

(ii) are of the same tumor type as a malignant tumor of a patient for treatment of whom the composition is intended;

(iii) are autologous to said patient; and

(iv) have been rendered incapable of growing in the body of a human upon injection therein; and

(b) an adjuvant;

wherein said composition elicits at least one of the following upon administration to said patient with the adjuvant: an inflammatory immune response against the tumor of said patient; a delayed-type hypersensitivity response against the tumor of said patient and activated T lymphocytes that infiltrate the tumor of said patient; and

repeating said administration at least six times at spaced apart intervals.

49. The composition of claim 43 wherein said tumor cells are selected from lung, colon, breast, kidney, and prostate tumor cells.

50. The composition of claim 43 wherein said hapten is selected from the group consisting of dinitrophenyl, trinitrophenyl, and N-iodoacetyl-N'-(5 sylfonic 1-naphtyl) ethylene diamine.

51. The composition of claim 43 wherein said hapten is dinitrophenyl.

52. The composition of claim 43 further comprising an adjuvant.

53. The composition of claim 52 wherein said adjuvant is Bacillus Calmette-Guerin.

54. The composition of claim 43 further comprising a carrier.

55. The composition of claim 54 wherein said carrier is selected from the group consisting of saline solution and culture medium.

56. The method of claim 44 wherein said tumor cells are selected from lung, colon, breast, kidney, and prostate tumor cells.

57. The method of claim 44, wherein said malignant tumor is from a cancer selected from the group consisting of lung cancer, colon cancer, breast cancer, kidney cancer, and prostate cancer.

58. The method of claim 44 wherein said hapten is selected from the group consisting of dinitrophenyl, trinitrophenyl, and N-iodoacetyl-N'-(5-sulfonic 1-naphtyl) ethylene diamine.

59. The method of claim 44 wherein said hapten is dinitrophenyl.

60. The method of claim 44 further comprising administering a therapeutically effective amount of cyclophosphamide prior to administration of said composition.

61. The method of claim 60 wherein said therapeutically effective amount of cyclophosphamide comprises administering a dose of about 300 mg/M2 of cyclophosphamide prior to administration of said composition.

62. The method of claim 60 further comprising sensitizing the patient with a therapeutically effective amount of 1-fluoro-2,4-dinitrobenzene prior to administering cyclophosphamide.

64. The method of claim 44 wherein said adjuvant is Bacillus Calmette-Guerin.

65. The method of claim 47 wherein said tumor cells are selected from melanoma, lung, colon, breast, kidney, and prostate tumor cells.

66. The method of claim 47, wherein said malignant tumor is from a cancer selected from the group consisting of melanoma cancer, lung cancer, colon cancer, breast cancer, kidney cancer, and prostate cancer.

67. The method of claim 47 wherein said hapten is selected from the group consisting of dinitrophenyl, trinitrophenyl, and N-iodoacetyl-N'-(5-sulfonic 1-naphtyl) ethylene diamine.

68. The method of claim 47 wherein said hapten is dinitrophenyl.

69. The method of claim 47 further comprising administering a therapeutically effective amount of cyclophosphamide prior to administration of said composition.

70. The method of claim 47, further comprising administering a therapeutically effective amount of cyclophosphamide prior to the first administration of said composition.

71. The method of claim 69 wherein said therapeutically effective amount of cyclophosphamide comprises administering a dose of about 300 mg/M2 of cyclophosphamide prior to administration of said composition.

72. The method of claim 47 further comprising sensitizing the patient with a therapeutically effective amount of 1-fluoro-2,4-dinitrobenzene prior to administering cyclophosphamide.

74. The method of claim 47 wherein said adjuvant is Bacillus Calmette-Guerin.

75. The method of claim 47 wherein said administration prolongs survival of said patient.

76. The method of claim 44, wherein said administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8 + CD4.

77. The method of claim 47, wherein said administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8 + CD4.

Exhibit 1

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
08/203,004	02/28/94	BERD	D 1225/0C674

08/203,004 02/28/94 Brief

DUE: July 29 2001

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Attorney

EXAMINER	
ART UNIT	PAPER NUMBER
1642	48

DATE MAILED: 07/05/01

12/29/01

Below is a communication from the EXAMINER in charge of this application

COMMISSIONER OF PATENTS AND TRADEMARKS

ADVISORY ACTION

☑ THE PERIOD FOR RESPONSE:

a) ☐ is extended to run _____ or continues to run _____ from the date of the final rejection

b) ☐ expires three months from the date of the final rejection or as of the mailing date of this Advisory Action, whichever is later. In no event however, will the statutory period for the response expire later than six months from the date of the final rejection.

Any extension of time must be obtained by filing a petition under 37 CFR 1.136(a), the proposed response and the appropriate fee. The date on which the response, the petition , and the fee have been filed is the date of the response and also the date for the purposes of determining the period of extension and the corresponding amount of the fee. Any extension fee pursuant to 37 CFR 1.17 will be calculated from the date of the originally set shortened statutory period for response or as set forth in b) above.

☑ Appellant's Brief is due in accordance with 37 CFR 1.192(a).

☐ Applicant's response to the final rejection, filed June 2, 2001 has been considered with the following effect, but it is not deemed to place the application in condition for allowance:

1. ☐ The proposed amendments to the claim and /or specification will not be entered and the final rejection stands because:

   a. ☐ There is no convincing showing under 37 CFR 1.116(b) why the proposed amendment is necessary and was not earlier presented.

   b. ☐ They raise new issues that would require further consideration and/or search. (See Note).

   c. ☐ They raise the issue of new matter. (See Note).

   d. ☐ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal.

   e. ☐ They present additional claims without cancelling a corresponding number of finally rejected claims.

NOTE: _____

2. ☐ Newly proposed or amended claims _____ would be allowed if submitted in a separately filed amendment cancelling the non-allowable claims.

3. ☑ ~~Upon the filing an appeal, the proposed amendment~~ ☐ ~~will be entered~~ ☐ ~~will not be entered~~ and the status of the claims will be as follows:

The AMENDMENT has been entered

Claims allowed: None

Claims objected to: None

Claims rejected: 43, 44, 47, 49-62, 64-72 + 74-77

However;

☑ Applicant's response has overcome the following rejection(s): Rejection of Cl 43, 49-51, 54-55 under 112 1st par.

4. ☐ The affidavit, exhibit or request for reconsideration has been considered but does not overcome the rejection because _____

5. ☑ The affidavit or exhibit will not be considered because applicant has not shown good and sufficent reasons why it was not earlier presented.

☐ The proposed drawing correction ☑ has ☐ has not been approved by the examiner.

☐ Other

Susan Ungar

1. The Amendment After-Final filed June 2, 2001 (Paper No. 46) in response to the Office Action of November 29, 2000 (Paper No. 44) is acknowledged and has been entered. Previously pending claims 43 and 47 have been amended. Claims 43, 44, 47, 49-62, 64-72 and 74-77 are currently being examined.

Claim Rejections - 35 USC § 103

2. Claims 47, 65-72 and 74-77 remain rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Sections 10-11, pages 8-15, Paper No. 41, Sections 5-6, pages 2-4 and in Paper No. 44, Sections 5 and 6, pages 3-5.

Applicant argues that (a) Examiner gives more weight to the Berd '89 abstract than one of ordinary skill at the time the invention would have given it and the Braun Declaration sets forth that Berd '89 does not describe a successful immunotherapy for melanoma, (b) Applicant details the three basic criteria that must be met to establish a *prima facie* case for obviousness under 35 USC 103 and states that since Berd '89 fails to provide any expectation of success the reference is completely irrelevant and no other reference provided makes up for this flaw, (c) Applicant reiterates the deficiencies of Murphy, Berd '89 Geczy and Antibody Patents and the combination thereof. The arguments have been considered but have not been found persuasive (a') for the reasons previously set forth in Paper No. 41, Section 7, pages 4-5. The Braun Declaration has not been considered because Applicant has not shown good and sufficient reasons why it was not earlier presented, (b') the Berd '89 reference is not irrelevant for the reasons set forth previously and above, (c') the arguments are not persuasive for the reasons previously set forth

3. Claims 43, 44, 47, 49-62, 64-72, and 74-77 remain rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Section 12, pages 15-18, Paper No. 41, Section 7, pages 4-5 and in Paper No. 44, Section 7, pages 5-6.

Applicant reiterates the arguments drawn to Wiseman and the arguments drawn to the combination of Berd '89, The Antibody Patents and Geczy. The arguments have been considered but have not been found persuasive for the reasons previously set forth.

4. Claims 43, 44, 47, 49-62, 64-72, and 74-77 remain rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Section 13, pages 18-21, Paper No. 41, Section 8, page 6 and in Paper No. 44, Section 8, page 6.

Applicant reiterates arguments drawn to Berd '83. The arguments have been considered but have not been found persuasive for the reasons previously set forth in Paper Nos 36, 41 and 44.

5. Claims 43, 44, 47, 49-62, 64-72, and 74-77 remain rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Section 14, pages 21-25, Paper No. 41, Section 9, page 7 and in Paper No. 44, Section 9, page 7.

Applicant reiterates the arguments previously set forth. The arguments have been considered but have not been found persuasive for the reasons previously set forth in Paper Nos 36, 41 and 44.

6. All other objections and rejections recited in Paper No. 44 are withdrawn.

SUSAN UNGAR, PH.D
PRIMARY EXAMINER

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

08/203004

APPLICATION NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.

EXAMINER	
ART UNIT	PAPER NUMBER
1642	

DATE MAILED:

INTERVIEW SUMMARY

All participants (applicant, applicant's representative, PTO personnel):

(1) S. UNGAR (3) _____

(2) P. FEHLNER (4) _____

Date of Interview 6/13/01

Type: ☑ Telephonic ☐ Personal (copy is given to ☐ applicant ☐ applicant's representative).

Exhibit shown or demonstration conducted: ☐ Yes ☐ No If yes, brief description: _____

Agreement ☐ was reached. ☐ was not reached.

Claim(s) discussed: All pending.

Identification of prior art discussed: _____

Description of the general nature of what was agreed to if an agreement was reached, or any other comments:
Rec'd AF + ~~DON~~ reviewing for patentable Material
will be back to AMY w/ a few days

(A fuller description, if necessary, and a copy of the amendments, if available, which the examiner agreed would render the claims allowable must be attached. Also, where no copy of the amendments which would render the claims allowable is available, a summary thereof must be attached.)

1. ☐ It is not necessary for applicant to provide a separate record of the substance of the interview.

Unless the paragraph above has been checked to indicate to the contrary. A FORMAL WRITTEN RESPONSE TO THE LAST OFFICE ACTION IS NOT WAIVED AND MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a response to the last Office action has are ready been filed, APPLICANT IS GIVEN ONE MONTH FROM THIS INTERVIEW DATE TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW.

2. ☐ Since the Examiner's interview summary above (including any attachments) reflects a complete response to each of the objections, rejections and requirements that may be present in the last Office action, and since the claims are now allowable, this completed form is considered to fulfill the response requirements of the last Office action. Applicant is not relieved from providing a separate record of the interview unless box 1 above is also checked.

Examiner Note: You must sign this form unless it is an attachment to another form.

FORM PTOL-413 (REV.1-96)

Attachment for PTO-948 (Rev. 03/01, or earlier)
6/18/01

The below text replaces the pre-printed text under the heading, "Information on How to Effect Drawing Changes," on the back of the PTO-948 (Rev. 03/01, or earlier) form.

INFORMATION ON HOW TO EFFECT DRAWING CHANGES

1. Correction of Informalities -- 37 CFR 1.85

New corrected drawings must be filed with the changes incorporated therein. Identifying indicia, if provided, should include the title of the invention, inventor's name, and application number, or docket number (if any) if an application number has not been assigned to the application. If this information is provided, it must be placed on the front of each sheet and centered within the top margin. If corrected drawings are required in a Notice of Allowability (PTOL-37), the new drawings MUST be filed within the THREE MONTH shortened statutory period set for reply in the Notice of Allowability. Extensions of time may NOT be obtained under the provisions of 37 CFR 1.136(a) or (b) for filing the corrected drawings after the mailing of a Notice of Allowability. The drawings should be filed as a separate paper with a transmittal letter addressed to the Official Draftsperson.

2. Corrections other than Informalities Noted by Draftsperson on form PTO-948.

All changes to the drawings, other than informalities noted by the Draftsperson, MUST be made in the same manner as above except that, normally, a highlighted (preferably red ink) sketch of the changes to be incorporated into the new drawings MUST be approved by the examiner before the application will be allowed. No changes will be permitted to be made, other than correction of informalities, unless the examiner has approved the proposed changes.

Timing of Corrections

Applicant is required to submit the drawing corrections within the time period set in the attached Office communication. See 37 CFR 1.85(a).

Failure to take corrective action within the set period will result in ABANDONMENT of the application.

Exhibit 2

AL

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

VB

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
08/202,004	02/28/94	BERD	D 1225/0C674

EXAMINER	
UNGAR, S	
ART UNIT	**PAPER NUMBER**
1642	44

HM22/1129

DARBY & DARBY PC
805 THIRD AVENUE
NEW YORK NY 10022

FINAL

DUE: February 28, 2001
Docketed on 12/6 by DA for
Docketed without file ☐
Attorney

DATE MAILED: 11/29/00
5-29-01

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

2000 DEC -2 A 10:01
RECEIVED

Office Action Summary	Application No. 08/203,004	Applicant(s) Berd
	Examiner Unger	Group Art Unit 1642

☒ Responsive to communication(s) filed on *Sep 27, 2000*.

☒ This action is **FINAL**.

☐ Since this application is in condition for allowance except for formal matters, **prosecution as to the merits is closed** in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11; 453 O.G. 213.

A shortened statutory period for response to this action is set to expire *three* month(s), or thirty days, whichever is longer, from the mailing date of this communication. Failure to respond within the period for response will cause the application to become abandoned. (35 U.S.C. § 133). Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

Disposition of Claims

☒ Claim(s) *43, 44, 47, 49-62, 64-72, and 74-77* is/are pending in the application.

Of the above, claim(s) _____ is/are withdrawn from consideration.

☐ Claim(s) _____ is/are allowed.

☒ Claim(s) *43, 44, 47, 49-62, 64-72, and 74-77* is/are rejected.

☐ Claim(s) _____ is/are objected to.

☐ Claims _____ are subject to restriction or election requirement.

Application Papers

☐ See the attached Notice of Draftsperson's Patent Drawing Review, PTO-948.

☐ The drawing(s) filed on _____ is/are objected to by the Examiner.

☐ The proposed drawing correction, filed on _____ is ☐approved ☐disapproved.

☐ The specification is objected to by the Examiner.

☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119

☐ Acknowledgement is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).

☐ All ☐ Some* ☐ None of the CERTIFIED copies of the priority documents have been

☐ received.

☐ received in Application No. (Series Code/Serial Number) _____.

☐ received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

*Certified copies not received: _____.

☐ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e).

Attachment(s)

☐ Notice of References Cited, PTO-892

☐ Information Disclosure Statement(s), PTO-1449, Paper No(s). _____

☐ Interview Summary, PTO-413

☐ Notice of Draftsperson's Patent Drawing Review, PTO-948

☐ Notice of Informal Patent Application, PTO-152

--- SEE OFFICE ACTION ON THE FOLLOWING PAGES ---

1. The Amendment filed September 27, 2000 (Paper No. 43) in response to the Office Action of March 22, 2000 (Paper No. 41) is acknowledged and has been entered. Previously pending claims 54, 55, 57, 66 and 70 have been amended. Claims 43, 44, 47 and 49-62, 64-72 and 74-77 are currently being examined.

2. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

3. The following rejections are being maintained:

Claim Rejections - 35 USC § 112

4. Claims 43, 49-51, 54-55 remain rejected under 35 USC 112, first paragraph for the reasons previously set forth in Paper No. 41, Section 10, pages 7-8.

Applicant argues that the composition is a useful component of a vaccine and that the specification enables a haptenized tumor cell composition in combination with an adjuvant and that for that reason both components must be enabled. Applicant further cites case law to demonstrate that products are useful if they serve as starting materials in producing other materials which are directly useful and therefore Examiner errs in interpreting these claims as requiring adjuvant. The argument has been considered but has not been found persuasive because the claims are not drawn to a component of a vaccine that upon addition of an adjuvant will elicit an immune response but rather specifically recite the limitation that upon injection the composition (without adjuvant) elicits an inflammatory immune response. For the reasons previously set forth, without an adjuvant the claim is not enabled. Applicant's arguments have not been found persuasive and the rejection is maintained.

Claim Rejections - 35 USC § 103

5. Claims 47 and 65-72 and 74-77 remain rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Section 10, pages 8-12 and in Paper No. 41, Section 5, pages 2 and 3.

Applicant argues (a) in order for a combination of prior art references to suggest a claimed invention, an objective teaching must exist in the prior art that would lead a skilled artisan to combine its teaching; (b) it is impermissible to use hindsight reconstruction to pick and choose among isolated disclosures in the prior art to deprecated the claimed invention, (c) Applicant takes issue with Examiner's contention that Applicant has argued and discussed the references individually without clearly addressing the combined teachings since each reference must be considered in its sentirely and then evaluation must be made as to whether the reference can be properly combined with the others and whether the combination suggests the claimed invention with a reasonable expecation of success (d) Applicant discusses the individual references, (e) Applicant reiterates the arguments drawn to the rejection of claims 47 and 65-76 in Paper No. 36, Section 10, pages 8-12.

The arguments have been considered but have not been found persuasive (a') for the reasons previously set forth in Paper No. 51 on page 3, it is suggested that Applicant review In re Keller, 642 F.2d 413, 208 USPQ 871 (CCPA 1981), (b') Some degree of hindsight is permissible in making rejections under 35 USC 103 since it must be recognized that any judgment on obviousness is in a sense necessarily a reconstruction based upon hindsight reasoning. But, so long as it takes

into account only knowledge which was within the level of ordinary skill at the time the claimed invention was made, and does not include knowledge gleaned only from the applicant's disclosure, such a reconstruction is proper. In re McLaughlin , 443 F.2d 1392, 170 USPQ 209 (CCPA 1971). It is clear that a method for treating melanoma comprising sensitizing with DNCB, administering a therapeutically effective amount of cyclophosphamide and administering a therapeutically effective amount of autologous, irradiated DNP-conjugated melanoma cells mixed with BCG was well known in the art as taught by Murphy et al and that immunization schedules wherein antigen is administered on a schedule of at least six times at spaced intervals was conventional in the art as taught by the Antibody Patents cited on Page 10 of Paper No. 36 and that a dose of 300 mg/M^2 was successfully administered prior to autologous irradiated, DNP-conjugated melanoma cells as taught by Berd et al and the equivalence of halogenated dinitrobenzenes for the elicitation of delayed hypersensitivity was known in the art as taught by Geczy et al. Given the information known in the art, the combined references teach not only the suggestion but also the means and motivation to successfully treat a malignant tumor in a human patient as claimed, (c') upon review and reconsideration it is found that, in Examiner's view, Applicant did not clearly address the combined teachings of the references, (d') and (e') Applicant's arguments are not persuasive for the reasons previously set forth. Applicant's arguments have not been found persuasive and the rejection is maintained.

6. Claims 47 and 65-72 and 74-77 remain rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Section 11, pages 12-15 and in Paper No. 41, Section 6, page 4.

Applicant argues that for the reasons set forth above the rejection is in error and should be withdrawn. The argument has been considered but is not found persuasive for the reasons set forth above. Applicant's arguments have not been found persuasive and the rejection is maintained.

7. Claims 43, 44, 47 and 49-62, 64-72 and 74-77 remain rejected for the reasons previously set forth in Paper No. 36, Section 12, pages 15-18 and in Paper No. 41, Section 7, pages 4-5.

Applcant argues that (a) it is not expected that vaccines using other types of tumor cells would behave in a mechanistically similar manner to the melanoma vaccine described in Berd et al since Hanna et al teaches a method for treatment of human colon cancer using a vaccine made from irradiated human tumor cells and Hoover teaches that when comparing vaccines made from irradiated autologous cells of colon cancer patients and rectal cancer patients, only the colon cancer patients received benefit from the vaccine, (b) Applicant again argues that Wiseman does not supply the missing teaching and the "preliminary" nature of the results of Berd et al.

The arguments have been considered but have not been found persuasive because (a') Wiseman clearly showed that autologous irradiated melanoma, lung, colon and kidney cancer cells were successfully used for successful immunological treatment of those cancers and it would have been expected that these cell types,

already known in the art to be useful as immunogenic cancer treatments would be successfully substituted for the melanoma cells of Berd et al in order to treat the other cancer types. The fact that a single cancer cell type is not immunogenic is not relevant in view of the demonstrated efficacy of the cell types of Wiseman, (b') the arguments are not persuasive for the reasons previously set forth.Applicant's arguments have not been found persuasive and the rejection is maintained.

8. Claims 43, 44, 47 and 49-62, 64-72 and 74-77 remain rejected for the reasons previously set forth in Paper No. 36, Section 13, pages 18-21 and in Paper No. 41, Section 8, page 6.

Applicant argues that Berd '83 does not provide an incentive to prepare a haptenized tumor cell vaccine or methods of treating cancer using said vaccine. The argument has been noted but has not been found persuasive for the reasons previously set forth. Applicant is arguing this reference individually without clearly addressing the combined teachings. It must be remembered that the references are relied upon in combination and are not meant to be considered separately as in a vacuum. It is the combination of all of the cited and relied upon refefrences which made up the state of teh art with regard to the claimed invention. Applicant's claimed invention fails to patentably distinguish over the state of the art represented by the cited references taken in combination. In re Young, 403 F.2d 754, 159 USPQ 725 (CCPA 1968); In re Keller 642 F.2d 413,208 USPQ 871 (CCPA 1981). Applicant's arguments have not been found persuasive and the rejection is maintained.

9. Claims 43, 44, 47 and 49-62, 64-72 and 74-77 remain rejected for the reasons previously set forth in Paper No. 36, Section 14, pages 21-25 and in Paper No. 41, Section 9, page 7.

Applicant reiterates the arguments previously set forth. The arguments have been considered but have not been found persuasive for the reasons previously set forth. Applicant's arguments have not been found persuasive and the rejection is maintained.

10. All other objections and rejections recited in Paper No. 41 are withdrawn.

11. No claims allowed.

12 **THIS ACTION IS MADE FINAL.** Applicant is reminded of the extension of time policy as set forth in 37 C.F.R. § 1.136(a).

A SHORTENED STATUTORY PERIOD FOR RESPONSE TO THIS FINAL ACTION IS SET TO EXPIRE THREE MONTHS FROM THE DATE OF THIS ACTION. IN THE EVENT A FIRST RESPONSE IS FILED WITHIN TWO MONTHS OF THE MAILING DATE OF THIS FINAL ACTION AND THE ADVISORY ACTION IS NOT MAILED UNTIL AFTER THE END OF THE THREE-MONTH SHORTENED STATUTORY PERIOD, THEN THE SHORTENED STATUTORY PERIOD WILL EXPIRE ON THE DATE THE ADVISORY ACTION IS MAILED, AND ANY EXTENSION FEE PURSUANT TO 37 C.F.R. § 1.136(a) WILL BE CALCULATED FROM THE MAILING DATE OF THE ADVISORY ACTION. IN NO EVENT WILL THE STATUTORY PERIOD FOR RESPONSE EXPIRE LATER THAN SIX MONTHS FROM THE DATE OF THIS FINAL ACTION.

13. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Susan Ungar, PhD whose telephone number is

(703) 305-2181. The examiner can normally be reached on Monday through Friday from 7:30am to 4pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Anthony Caputa, can be reached at (703) 308-3995. The fax phone number for this Art Unit is (703) 308-4242.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group receptionist whose telephone number is (703) 308-0196.

Effective, February 7, 1998, the Group and/or Art Unit location of your application in the PTO has changed. To aid in correlating any papers for this application, all further correspondence regarding this application should be directed to Group Art Unit 1642.

Susan Ungar
Primary Patent Examiner
November 28, 2000

Exhibit 3

PFF

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

VB

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
08/203,004	02/28/94	BERD D	1225/0C674

EXAMINER	
UNGAR, S	
ART UNIT	**PAPER NUMBER**
1642	41

HM12/0322

DARBY & DARBY PC
805 THIRD AVENUE
NEW YORK NY 10022

DUE: June 22, 2000
Docketed on 3/27 by DP for
Docketed without file ☐
Attorney PFF

DATE MAILED: 03/22/00
9-22-00

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

Office Action Summary	Application No. 08/203,004	Applicant(s) Berd
	Examiner Ungar	Group Art Unit 1642

☒ Responsive to communication(s) filed on *Jan 14, 2000*.

☐ This action is **FINAL**.

☐ Since this application is in condition for allowance except for formal matters, **prosecution as to the merits is closed** in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11; 453 O.G. 213.

A shortened statutory period for response to this action is set to expire *three* month(s), or thirty days, whichever is longer, from the mailing date of this communication. Failure to respond within the period for response will cause the application to become abandoned. (35 U.S.C. § 133). Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

Disposition of Claims

☒ Claim(s) *43, 44, 47, and 49-77* is/are pending in the application.

Of the above, claim(s) _____ is/are withdrawn from consideration.

☐ Claim(s) _____ is/are allowed.

☒ Claim(s) *43, 44, 47, and 49-77* is/are rejected.

☐ Claim(s) _____ is/are objected to.

☐ Claims _____ are subject to restriction or election requirement.

Application Papers

☐ See the attached Notice of Draftsperson's Patent Drawing Review, PTO-948.

☐ The drawing(s) filed on _____ is/are objected to by the Examiner.

☐ The proposed drawing correction, filed on _____ is ☐approved ☐disapproved.

☐ The specification is objected to by the Examiner.

☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119

☐ Acknowledgement is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).

☐ All ☐ Some* ☐ None of the CERTIFIED copies of the priority documents have been

☐ received.

☐ received in Application No. (Series Code/Serial Number) _____.

☐ received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

*Certified copies not received: _____.

☐ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e).

Attachment(s)

☐ Notice of References Cited, PTO-892

☐ Information Disclosure Statement(s), PTO-1449, Paper No(s). _____

☐ Interview Summary, PTO-413

☐ Notice of Draftsperson's Patent Drawing Review, PTO-948

☐ Notice of Informal Patent Application, PTO-152

--- SEE OFFICE ACTION ON THE FOLLOWING PAGES ---

1. The Amendment filed November 4, 1999 (Paper No. 38) in response to the Office Action of April 28, 1999 (Paper No. 36) is acknowledged and has been entered. The Amendment (Paper No. 39) and the Terminal Disclaimer (Paper No. 40) filed January 14, 2000, in response to the telephone interview of January 13, 2000 (Paper No. 38.5) have been entered Previously pending claims 63 and 73 have been canceled, claims 44, 47, 64, 74 and 76 have been amended and new claim 77 has been added. Claims 43, 44, 47 49-62, 64-72 and 74-77 are currently being examined.

2. The Terminal Disclaimer filed January 14, 2000 is acceptable.

3. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

4. The following rejections are being maintained:

Claim Rejections - 35 USC § 103

5. Claims 47 and 65-76 remain rejected for the reasons previously set forth in Paper No. 36, Sections 10 , pages 8-12.

Applicant argues that (a) the legal test for obviousness cannot be established by combining teachings of the prior art absent some teaching or suggestion supporting the combination. Under section 103, teachings of references can be combined only if there is some suggestion or incentive to do so, (b) Murphy and or Berd fail to teach a method of treatment for non-melanoma malignant tumors in a human patient, (c) the antibody patents which merely teach conventional methods for generating antibodies provide no missing teaching, (d) Geczy fails to provide any teaching pertinent to the claimed compositions and methods.

The arguments have been considered but have not been found persuasive because (a') contrary to the assertion of Applicant, the test for obviousness is not that the claimed invention must be expressly suggested in any one or all of the references; but rather the test is what the combined teachings of the references would have suggested to those of ordinary skill in the art, (b') Applicant is arguing limitations not recited in the claims as currently constituted. The claims are drawn to a method of treating a malignant tumor in a human patient and as broadly written, read on the treatment of melanoma (b'-d') Applicant has argued and discussed the references individually without clearly addressing the combined teachings. It must be remembered that the references are relied upon in combination and are not meant to be considered separately as in a vacuum. It is the combination of all of the cited and relied upon references which made up the state of the art with regard to the claimed invention. Applicant's claimed invention fails to patentably distinguish over the state of the art represented by the cited references taken in combination. In re Young, 403 F.2d 754, 159 USPQ 725 (CCPA 1968); In re Keller 642 F.2d 413,208 USPQ 871 (CCPA 1981), (c') multiple immunizations are conventional in the art for producing all forms of immune response, (d') Geczy clearly teach the equivalence of CDNB and FDNB. The references teach not only the suggestion but also the means and motivation to successfully treat melanoma by sensitizing with FDNB and administering cyclophosphamide prior to administering DNP-conjugated tumor cells and an adjuvant, which treatment results in the claimed T-cell dependent responses. Applicant's arguments have not been found persuasive and the rejection is maintained.

6. Claims 47 and 65-76 remain rejected for the reasons previously set forth in Paper No. 36, Sections 11 , pages 12-15.

Applicant argues that, for the reasons set forth above, the rejection is in error. The argument has been considered but has not been found persuasive for the reasons set forth above. Applicant's arguments have not been found persuasive and the rejection is maintained.

7. Claims 43, 44, 47 and 49-76 remain rejected for the reasons previously set forth in Paper No. 36, Sections 12 , pages 15-18.

Applicant argues that (a) there is no reasonable expectation of successfully implementing the vaccination program described with respect to melanoma in Berd to other tumor types because this reference provides "preliminary" results that "may represent a significant advance in the immunotherapy of human melanoma and thus it lacks any reasonable expectation of an effective treatment for tumors in general or even melanoma in particular, (b)Wiseman does not supply the missing teaching and teaches an alternative form of immunotherapy that depends on the route of administration, (c) even if combined, the lack of any reasonable expectation of success from the disclosure of Berd precludes determining that the invention is obvious.

The arguments have been considered but have not been found persuasive because (a) Berd et al clearly teach that treatment of melanoma patients with autologous vaccine preceded by low dose CY induces DTH to melanoma cells and regression of metastatic tumors. The reference is specifically drawn to increasing the efficiency of the process by sensitizing with DNCB and immunizing with tumor

cells conjugated to DNP. Clearly, one would have an expectation of success since it was already known that immunization with tumor cells alone, after pretreatment with CY resulted in regression of metastatic tumors. Further, it was clearly demonstrated that a patient developed erthema, followed by ulceration and drainage of necrotic material in > 50 large dermal metastasis and that at the time of publication, some of the metastasis were beginning to regress, Further the reference specifically teaches that tumors were infiltrated with activated T lymphocytes, that the tumor masses developed a striking inflammatory response and that the patients developed DTH, (b') regardless of the route of administration, Wiseman clearly teaches that treatment of patients with lung, colon and kidney cancer with autologous tumor cell vaccine preceded by low dose CY leads to prolonged survival. It would have been expected that vaccines using other types of tumor cells, shown to effectively treat cancer, would behave in a mechanistically similar manner to the melanoma vaccine described in Berd et al where it was shown that administration of the DNP-conjugated reagent led to DHT against melanoma cells, infiltration of the tumors by activated T lymphocytes, inflammation in the tumor masses, especially in view of the fact that there is no teaching of any no distinguishing features of melanoma tumor cells which would lead one to expect that there would be a difference in the immune response to this type of tumor cell in particular, (c') Berd et al clearly demonstrate the successful use of the composition and the method. Applicant's arguments have not been found persuasive and the rejection is maintained.

8. Claims 43, 44, 47 and 49-76 remain rejected for the reasons previously set forth in Paper No. 36, Sections 13 , pages 18- 21.

Applicant argues that Berd, 1983 is cumulative to the teaching of Berd, 1989 and that Berd 1983 does not supply any of the other missing teachings that are not supplied by the combination of Berd, the antibody Patents and Geczy and that in particular, the reference does not provide any teaching concerning a haptenized tumor cell vaccine or methods of treating cancer using such a vaccine. The argument has been considered but has not been found persuasive for the reasons drawn to the lack of persuasiveness of Applicant's arguments drawn to Berd, the antibody Patents and Geczy disclosed above and further because, Berd, 1983 is cited because it is drawn to treatment of breast cancer patients with autologous vaccine. The substitution of the breast cancer cells of Berd 1983 for the melanoma cells of Berd, 1989 in the method and composition of Berd 1989 was *prima facie* obvious for the reasons set forth in Paper No. 36. Applicant has argued and discussed the references individually without clearly addressing the combined teachings. It must be remembered that the references are relied upon in combination and are not meant to be considered separately as in a vacuum. It is the combination of all of the cited and relied upon references which made up the state of the art with regard to the claimed invention. Applicant's claimed invention fails to patentably distinguish over the state of the art represented by the cited references taken in combination. In re Young, 403 F.2d 754, 159 USPQ 725 (CCPA 1968); In re Keller 642 F.2d 413,208 USPQ 871 (CCPA 1981). Applicant's arguments have not been found persuasive and the rejection is maintained.

9. Claims 43, 44, 47 and 49-76 remain rejected for the reasons previously set forth in Paper No. 36, Sections 14 , pages 21-25.

Applicant argues that (a) the deficiencies of Berd, the antibody Patents and Geczy have been addressed above, (b) Sanda and Moody fail to supply the missing teachings and propose an alternative cancer therapy and neither references provides any motivation to decorate the tumor cells with hapten in order to elicit an effective immune response. The arguments have been considered but have not been found persuasive because (a') for the reasons set forth above, (b') the combined references make the invention obvious for the reasons set forth previously and in particular Berd supplies the motivation to "decorate the tumor cells with hapten" in order to elicit an effective immune response, see above.

New Grounds of Rejection

Claim Rejections - 35 USC § 112

10. Claims 43, 49-51, 54-55 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for a composition comprising the claimed tumor cell conjugate and an adjuvant, does not reasonably provide enablement for a composition comprising the claimed tumor cell conjugate alone. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention commensurate in scope with these claims.

The claims are drawn to treatment of malignant tumors with a composition comprising a hapten conjugated to a tumor cell. The specification teaches a melanoma vaccine administered with BCG and describes immune responses to the

melanoma vaccine administered with BCG (page 19-43) and specifically teaches in the sentence bridging pages 27-28 that "all vaccines were DNP-conjugated and mixed with BCG". It appears that the inclusion of an adjuvant may be a critical step since Livingstone et al (of record) disclosed that in a melanoma vaccine using the GM2 ganglioside, antibody responses were not induced unless BCG was added to the purified GM2 vaccine (p. 2913, paragraph bridging columns 1 and 2). Livingstone et al also state that "adjuvants ..... were important factors in the mouse studies and results of the present human trials indicate their importance in melanoma patients". Further, Hoover et al, of record, also used BCG as an adjuvant in a colorectal cancer vaccine and states that the correct amount of the appropriate adjuvant was a critical condition of the success of the immunotherapy (p. 1242, col 1, para 2). Based on the teachings above and in the specification one of skill in the art would not expect that the claimed composition could be used as contemplated for the treatment of malignant tumors without specifically including an adjuvant as demonstrated in the specification. In view of the above, one of skill in the art would be forced into undue experimentation to practice the claimed invention.

11. Claims 70 is rejected under 35 USC 112, first paragraph, as the specification does not contain a written description of the claimed invention. The limitation of cyclophosphamide administration "only" prior to the first administration of said composition has no clear support in the specification and the claims as originally filed. The specification teaches that cyclophosphamide is administered 3 days prior to each vaccine administration (p. 44, lines 26-27) but does not specifically state that the cyclophosphamide is only administered prior to vaccine. The subject matter

claimed in claims 47-58 broadens the scope of the invention as originally disclosed in the specification.

12. Claims 57, 66 and 70 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 57 is indefinite because there is no antecedent basis for the term "cancer" in claim 44 from which claim 57 depends.

Claim 66 is indefinite because there is no antecedent basis for the term "cancer" in claim 47 from which claim 66 depends.

Claim 70 is indefinite because there is no antecedent basis for the phrase "wherein said therapeutically effective amount of cyclophophamide" in claim 47 from which claim 70 depends. Further, the claim is indefinite as the term "cyclophophamide" appears to be a misspelling of the term cyclophosphamide.

Claim Rejections - 35 USC § 103

13. Claim 77 is rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Sections 10 , pages 8-12 and above drawn to the rejection of claims 47 and 65-76.

The claim is drawn to the method of claim 47 wherein said administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-.

The claim is obvious for the reasons previously set forth. Applicant's arguments are relevant to the instant rejection. The arguments drawn to the rejection of claims 47 and 65-76 under 35 USC 103 are relevant to the instant

rejection. The arguments have been considered but have not been found persuasive for the reasons set forth above.

14. Claim 77 is rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Sections 11 , pages 12-15 and above drawn to the rejection of claims 47 and 65-76.

The claim is drawn to the method of claim 47 wherein said administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-.

The claim is obvious for the reasons previously set forth. Applicant's arguments are relevant to the instant rejection. The arguments drawn to the rejection of claims 47 and 65-76 under 35 USC 103 are relevant to the instant rejection. The arguments have been considered but have not been found persuasive for the reasons set forth above.

15. Claim 77 is rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Sections 12 , pages 15-18 and above drawn to the rejection of claims 43, 44, 47 and 49-76.

The claim is drawn to the method of claim 47 wherein said administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-.

The claim is obvious for the reasons previously set forth. Applicant's arguments are relevant to the instant rejection. The arguments drawn to the rejection of claims 43, 44, 47 and 49-76 under 35 USC 103 are relevant to the

instant rejection. The arguments have been considered but have not been found persuasive for the reasons set forth above.

16. Claim 77 is rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Sections 13 , pages 18-21 and above drawn to the rejection of claims 43, 44, 47 and 49-76.

The claim is drawn to the method of claim 47 wherein said administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-.

The claim is obvious for the reasons previously set forth. Applicant's arguments are relevant to the instant rejection. The arguments drawn to the rejection of claims 43, 44, 47 and 49-76 under 35 USC 103 are relevant to the instant rejection. The arguments have been considered but have not been found persuasive for the reasons set forth above.

17. Claim 77 is rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Sections 14 , pages 21-25 and above drawn to the rejection of claims 43, 44, 47 and 49-76.

The claim is drawn to the method of claim 47 wherein said administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-.

The claim is obvious for the reasons previously set forth. Applicant's arguments are relevant to the instant rejection. The arguments drawn to the rejection of claims 43, 44, 47 and 49-76 under 35 USC 103 are relevant to the

instant rejection. The arguments have been considered but have not been found persuasive for the reasons set forth above.

18 All other objections and rejections recited in Paper No. 36 are withdrawn.

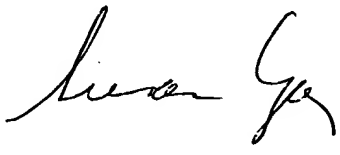
19. No claims allowed.

20. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Susan Ungar, PhD whose telephone number is (703) 305-2181. The examiner can normally be reached on Monday through Friday from 7:30am to 4pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Paula Hutzell, can be reached at (703) 308-4310. The fax phone number for this Art Unit is (703) 308-4242.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group receptionist whose telephone number is (703) 308-0196.

Effective, February 7, 1998, the Group and/or Art Unit location of your application in the PTO has changed. To aid in correlating any papers for this application, all further correspondence regarding this application should be directed to Group Art Unit 1642..



Susan Ungar
Primary Patent Examiner
March 20, 2000

Exhibit 4

08/203,004

APPLICATION NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO.
08/203,004	02/28/94	BLRD	1220/0067/A

EXAMINER	
UNGAR, S	

ART UNIT	PAPER NUMBER
1642	26

HM22/0429

DARBY & DARBY PC
805 THIRD AVENUE
NEW YORK NY 10022

DUE: July 28, 1999
Docketed on 5/3 by D for
Docketed without file ☐
Attorney

DATE MAILED: 04/28/99
10-28-99

This is a communication from the examiner in charge of your application.
COMMISSIONER OF PATENTS AND TRADEMARKS

OFFICE ACTION SUMMARY

☒ Responsive to communication(s) filed on 3/15/99

☐ This action is **FINAL**.

☐ Since this application is in condition for allowance except for formal matters, **prosecution as to the merits is closed** in accordance with the practice under *Ex parte Quayle*, 1935 D.C. 11; 453 O.G. 213.

A shortened statutory period for response to this action is set to expire 3 month(s), or thirty days, whichever is longer, from the mailing date of this communication. Failure to respond within the period for response will cause the application to become abandoned. (35 U.S.C. § 133). Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

Disposition of Claims

☒ Claim(s) 43-44,47-76 is/are pending in the application.
Of the above, claim(s) _____ is/are withdrawn from consideration.
☐ Claim(s) _____ is/are allowed.
☒ Claim(s) 43,44,47-76 is/are rejected.
☐ Claim(s) _____ is/are objected to.
☐ Claim(s) _____ are subject to restriction or election requirement.

Application Papers

☐ See the attached Notice of Draftsperson's Patent Drawing Review, PTO-948.
☐ The drawing(s) filed on _____ is/are objected to by the Examiner.
☐ The proposed drawing correction, filed on _____ is ☐ approved ☐ disapproved.
☐ The specification is objected to by the Examiner.
☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119

☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).

☐ All ☐ Some* ☐ None of the CERTIFIED copies of the priority documents have been

☐ received.
☐ received in Application No. (Series Code/Serial Number) _____.
☐ received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

*Certified copies not received: _____.

☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e).

Attachment(s)

☒ Notice of Reference Cited, PTO-892
☐ Information Disclosure Statement(s), PTO-1449, Paper No(s). _____
☐ Interview Summary, PTO-413

Notice of References Cited

Application No.	Applicant(s)		
08/203,004	Berd		
Examiner	Group Art Unit	Page 1 of 1	
Ungar	1642		

U.S. PATENT DOCUMENTS

	DOCUMENT NO.	DATE	NAME	CLASS	SUBCLASS
A	5,290,551	3/1/94	Berd	424	88
B	5,702,704	12/30/97	Bucala	424	137.1
C	5,6226,843	5/6/97	Skurkovich et al	424	140.1
D	5,008,183	4/16/91	Osther	435	5
E	4,232,001	11/4/80	Jensen et al	424	1
F					
G					
H					
I					
J					
K					
L					
M					

FOREIGN PATENT DOCUMENTS

	DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUBCLASS
N						
O						
P						
Q						
R						
S						
T						

NON-PATENT DOCUMENTS

	DOCUMENT (Including Author, Title, Source, and Pertinent Pages)	DATE
U	Sanda et al (J. Cellular Biochem. Suppl., 17, Part D, p. 120	1993
V	Wiseman et al (Western J. Med., 151:283-288)	1989
W	Berd et al (Proc. Am. Soc. Clin. Oncol., 2:56)	1983
X	Moody et al (J. Urol., 145:293A)	1991

S. Ungar 4/19/99

1. The Election filed March 15, 1999 (Paper No. 35) in response to the Office Action of February 1, 1999 (Paper No. 33) is acknowledged and has been entered. Claims 43, 44, 47 and 49-76 are pending in the application and Claims 24-35 have been withdrawn from further consideration by the examiner under 37 CFR 1.142(b) as being drawn to non-elected inventions. Claims 43, 44, 47 and 49-76 are currently under prosecution.

2. Applicant's election with traverse of the species of Melanoma and DNP in Paper No. 35 is acknowledged. The traversal is on the ground(s) that the no restriction of species was made in the grandparent application, Serial No. 07/520,649 filed May 8, 1990. The argument has been noted and found persuasive as drawn to the hapten species. However, the argument has not been found persuasive as drawn to the cancer type because a review of the cited application revealed that the reason that no election of species, drawn to types of cancers, was required was because no species were recited in the claims, further, the specification does not appear to be drawn to any type of cancer composition or methods of treatment, other than for melanoma. However, upon search of the elected species, it became clear that irradiated autologous cancer vaccines were well known in the art at the time the invention was made and that haptenization of tumor cell vaccines was well known, therefore there was no undue burden in the search of the species and the requirement is withdrawn. .

3. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

4. It is noted that a priority date of February 28, 1994 has been established for the instantly claimed application serial number 08/203,004 for all claims drawn to

breast, lung, colon, kidney or prostate tumors because the earlier filed applications do not disclose compositions comprising hapten modified tumor cell compositions or methods useful for the treatment of breast, lung, colon, kidney or prostate tumors. If applicant disagrees with any rejection set forth in this office action based on examiner's establishment of the priority date set forth above, applicant is invited to submit evidence pointing to the serial number, page and line where support can be found establishing an earlier priority date.

New Grounds of Rejection

Double Patenting

5. Claims 47, 65-71, 73, 74, 76 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1 and 2 of U.S. Patent No.5,290,551 . Although the conflicting claims are not identical, they are not patentably distinct from each other because they relate to the same inventive concept. The patented claims are generic to the instant claims and render the species claims obvious as they have all the characteristics of a vaccine useful for the treatment of a malignant tumor, melanoma, in a human patient comprising irradiated autologous melanoma cell conjugated to a hapten selected from the group including DNP mixed with an immunological adjuvant wherein said immunological adjuvant is BCG and a method of treating melanoma comprising administering cyclophosphamide followed by administration of a therapeutically effective amount of the claimed vaccine. The limitations recited drawn to eliciting an inflammatory immune response against a delayed hypersensitivity response against the tumor, activated T lymphocytes that infiltrate the tumor wherein the T lymphocytes are predominantly CD8+CD4- are inherent properties of the method since the

population that is treated and the method steps recited in US Patent No. No.5,290,551 are the same as those of the instant claims. Thus, because the method of the patent comprises the same method steps as claimed in the instant invention, with the same population, the claimed method is made obvious because the method will inherently lead to eliciting an inflammatory immune response against a delayed hypersensitivity response against the tumor, activated T lymphocytes that infiltrate the tumor wherein the T lymphocytes are predominantly CD8+CD4-. See Ex parte Novitski 26 USPQ 1389 (BPAI 1993). Further, the recitation of repetition of administration of the vaccine at least six times at spaced apart intervals in claim 47 does not render the claims unobvious because immunization schedules requiring administration of antigen at least six times at spaced intervals are conventional in the immunostimulation arts as demonstrated by the teaching in US Patent No. 5,702,704 (col 20, lines 12-26), No. 5,626,843 (col. 4, lines 28-38), No. 5,008,183 col 5 lines 48-54), No. 4,232,001 (col 5, lines 25-27) which all teach immunization with at least six booster injections of antigen. Although the teaching is drawn to the antibody art, it is well known that T cells must be stimulated in order to produce the detected antibodies.

Applicant's argument in Paper No. 32 drawn to claims 47 and 76 are relevant to the instant rejection. Applicant argues that claim 47 calls for treating cancer by administering human tumor cells by repeating administration at least 6 times and claim 76 claims that administration elicits predominantly CD4-CD8+ T lymphocytes and that these claims are not suggested by the claims of the '551 patent. The argument has been noted but has not been found persuasive for the reasons stated

above, that is that the 6 time repetition is conventional in the immunostimulation art and that the CD4-CD8+ limitation is an inherent property of the method.

Claim Rejections - 35 USC § 112

6. Claims 44, 47, 56-62, 65-72 and 75-76 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for a method of treating a malignant tumor in a human patient comprising administering the composition of claim 43 and BCG, does not reasonably provide enablement for treatment a malignant tumor in a human patient without administering an BCG in combination with the claimed composition. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention commensurate in scope with these claims.

The claims are drawn to treatment of malignant tumors with a composition comprising a hapten conjugated to a tumor cell. The specification teaches a melanoma vaccine administered with BCG and describes immune responses to the melanoma vaccine administered with BCG (page 19-43) and specifically teaches in the sentence bridging pages 27-28 that "all vaccines were DNP-conjugated and mixed with BCG". It appears that the inclusion of the adjuvant may be a critical step since Livingstone et al (of record) disclosed that in a melanoma vaccine using the GM2 ganglioside, antibody responses were not induced unless BCG was added to the purified GM2 vaccine (p. 2913, paragraph bridging columns 1 and 2). Livingstone et al also state that "adjuvants ..... were important factors in the mouse studies and results of the present human trials indicate their importance in melanoma patients". Hoover et al, of record, also used BCG as an adjuvant in a colorectal cancer vaccine and states that the correct amount of the appropriate adjuvant was a

critical condition of the success of the immunotherapy (p. 1242, col 1, para 2). Based on the teachings above and in the specification one of skill in the art would not expect that the claimed method would be effective in treating cancer with DNP-conjugated vaccines without specifically including BCG as demonstrated in the specification. In view of the above, one of skill in he art would be forced into undue experimentation to practice the claimed invention

7. Claim 73 is rejected under 35 USC 112, fourth paragraph because it does not further limit claim 47 from which it depends.

Claim Rejections - 35 USC § 102

8. Claim 76 is rejected under 35 U.S.C. § 102(a) as being anticipated by Murphy et al, of record.

The claim is drawn to a method of treating a malignant tumor in a patient comprising administering to the patient a composition comprising a therapeutically effective amount of tumor cells that are conjugated to a hapten, are of the same tumor type as a malignant tumor of the patient, are autologous to said patient and have been rendered incapable of growing in the body of a human upon injection therein, said administration eliciting T lymphocytes that infiltrate the tumor and that are predominantly CD8+CD4-.

Murphy et al teach a method for treating melanoma comprising administering a therapeutically effective amount of autologous, irradiated DNP-conjugated melanoma cells. Although the reference does not disclose, the elicitation of T lymphocytes that infiltrate the tumor and are predominantly CD8+CD4-, the method of the prior art comprises the same method steps as claimed in the instant invention, that is, administering to the patient a composition comprising a therapeutically

effective amount of tumor cells that are conjugated to a hapten, are of the same tumor type as a malignant tumor of the patient, are autologous to said patient and have been rendered incapable of growing in the body of a human upon injection to the same population, thus the claimed method is anticipated because the method will inherently lead to eliciting T lymphocytes that infiltrate the tumor and that are predominantly CD8+CD4-. See Ex parte Novitski 26 USPQ 1389 (BPAI 1993).

Applicant's arguments in Paper No. 32 drawn to the rejection of claim 76 under 35 USC 102(a) are relevant to the instant rejection. Applicant argues that new claim 76 calls for a method of treating cancer by administering hapten-conjugated human tumor cells wherein said administering elicits predominantly CD4-CD8+ T lymphocytes and the Murphy et al paper does not disclose this limitation. The argument has been noted but has not been found persuasive for the reasons disclosed above, that is that the elicitation of specific T lymphocytes that infiltrate the tumor is an inherent property of the method.

9. Claim 76 is rejected under 35 U.S.C. § 102(b) as being anticipated by Berd et al., Proc. AACR, 1989, 30:382, of record.

The claim is drawn to a method of treating a malignant tumor in a patient comprising administering to the patient a composition comprising a therapeutically effective amount of tumor cells that are conjugated to a hapten, are of the same tumor type as a malignant tumor of the patient, are autologous to said patient and have been rendered incapable of growing in the body of a human upon injection therein, said administration eliciting T lymphocytes that infiltrate the tumor and that are predominantly CD8+CD4-.

Berd et al teach a method for treating melanoma comprising administering a therapeutically effective amount of autologous, irradiated DNP-conjugated melanoma cells. Although the reference does not disclose, the elicitation of the T lymphocytes that infiltrate the tumor and are predominantly CD8+CD4-, the method of the prior art comprises the same method steps as claimed in the instant invention, that is, administering to the patient a composition comprising a therapeutically effective amount of tumor cells that are conjugated to a hapten, are of the same tumor type as a malignant tumor of the patient, are autologous to said patient and have been rendered incapable of growing in the body of a human upon injection, to the same population, thus the claimed method is anticipated because the method will inherently lead to eliciting T lymphocytes that infiltrate the tumor and that are predominantly CD8+CD4-. See Ex parte Novitski 26 USPQ 1389 (BPAI 1993).

Applicant's arguments in Paper No. 32 drawn to the rejection of claim 76 under 35 USC 102(b) are relevant to the instant rejection. Applicant argues that new claim 76 calls for a method of treating cancer by administering hapten-conjugated human tumor cells wherein said administering elicits predominantly CD4-CD8+ T lymphocytes and the Berd et al does not disclose this limitation. The argument has been noted but has not been found persuasive for the reasons disclosed above, that is that the elicitation of specific T lymphocytes that infiltrate the tumor is an inherent property of the method.

Claim Rejections - 35 USC § 103

10. Claims 47, 65-76 are rejected under 35 U.S.C. § 103 as being unpatentable over Murphy et al, of record draw to claim 76 in view of US Patent No. 5,702,704,

No. 5,626,843, No. 5,008,183 or No. 4,232,001 and Berd et al., Proc. AACR, 1989, 30:382, of record and Geczy et al, of record.

The claims are drawn to a method of treating a malignant tumor in a patient comprising administering to the patient a composition comprising a therapeutically effective amount of tumor cells that are conjugated to a hapten, are of the same tumor type as a malignant tumor of the patient, are autologous to said patient and have been rendered incapable of growing in the body of a human upon injection therein, said composition eliciting at least one of the following upon administration to said patient with an adjuvant: an inflammatory immune response against the tumor; a delayed-type hypersensitivity response against the tumor; and activated T lymphocytes that infiltrate the tumor and repeating said administration at least six times at spaced apart intervals, wherein said tumor cells are selected from a group including melanoma, wherein the treating is useful for treatment of cancer selected from a group including melanoma, wherein said hapten is selected from the group including DNP, wherein the method further comprises administering a therapeutically effective amount of cyclophosphamide prior to administration of said composition, wherein the dose is about 300 mg/M^2, wherein the patient is sensitized with 1-fluoro-2,4-nitrobenzene prior to administration of cyclophosphamide wherein the composition comprises an adjuvant, BCG, wherein the life of the patient is prolonged, wherein the administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-.

Murphy et al teach as set forth above and further teach a method for treating melanoma comprising sensitizing with DNCB, administering a therapeutically effective amount of cyclophosphamide and administering a therapeutically effective

amount of autologous, irradiated DNP-conjugated melanoma cells mixed with the adjuvant BCG. Murphy et al teach as set forth above but do not teach a method wherein the vaccine is boosted at least six times at spaced intervals, the administration of 300 mg/M^2 of cyclophosphamide, prior sensitization with 1-fluoro-2,4-nitrobenzene or eliciting at least one of the following upon administration to said patient with an adjuvant: an inflammatory immune response against the tumor; a delayed-type hypersensitivity response against the tumor; and activated T lymphocytes that infiltrate the tumor or wherein the administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-, wherein the life of the patient is prolonged.

US Patent No. 5,702,704 (col 20, lines 12-26), No. 5,626,843 (col. 4, lines 28-38), No. 5,008,183 col 5 lines 48-54), No. 4,232,001 (col 5, lines 25-27) all teach conventional immunization schedules wherein antigen administration is repeated at least six times at spaced intervals.

Berd et al teach a successful method of treating melanoma wherein a therapeutically effective amount of cyclophosphamide, 300 mg/M^2 of cyclophosphamide, is administered prior to autologous, irradiated, DNP-conjugated melanoma cells.

Geczy et al teach that halogenated dinitrobenzenes such as 1-chloro- and 1-fluoro-2,4-dinitrobenzene are commonly used to elicit delayed hypersensitivity (p. 189, para 1).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to combine the methods of Murphy et al and the cited patents because it is clearly conventional to repeat antigen administration at

least six times at spaced intervals. Although the teaching is drawn to the antibody art, it is well known that T lymphocytes must be stimulated in order to produce the detected antibodies. One of ordinary skill in the art at the time would have expected to successfully use the method with the conventional immunization schedule. Further, although the limitations drawn to an inflammatory immune response against the tumor, a delayed-type hypersensitivity response against the tumor and activated T lymphocytes that infiltrate the tumor wherein the administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4- are not recited in the reference, the method of the prior art comprises the same method steps as claimed in the instant invention, to treat the same population, thus the claimed method is anticipated because the method will inherently lead to an inflammatory immune response against the tumor, a delayed-type hypersensitivity response against the tumor and activated T lymphocytes that infiltrate the tumor wherein the administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-. See Ex parte Novitski 26 USPQ 1389 (BPAI 1993). Further, it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to use a dose of 300 mg/M^2 of cyclophosphamide in the method of Murphy et al because Berd et al teach that the dose is therapeutically effective in a method which uses the same haptenized melanoma cells with the same population of patients. One of ordinary skill in the art would have expected to successfully use the method of Murphy with a dosage of 300 mg/M^2 of cyclophosphamide because Berd et al demonstrated the successful use of the method. Finally, it would have been *prima facie* obvious to substitute DNFB for the DNCB of Murphy et al because Geczy et

al teach that halogenated dinitrobenzenes such a 1-chloro- and 1-fluoro-2,4, dinitrobenzenes are commonly used to elicit delayed hypersensitivity and are clearly closely related haptenic molecules which function to produce the same effects and are therefore functionally equivalent. Finally, as drawn to prolonged survival, the claimed method appears to be the same as that of the prior art method absent a showing of unobvious differences. The office does not have the facilities for examining and comparing applicant's method with the method of the prior art in order to establish that the method of the prior art does not possess the same material structural and functional characteristics of the claimed method. In the absence of evidence to the contrary, the burden is upon the applicant to prove that the method of the prior art does not result in prolonged survival of the patient and is functionally different than the method taught by the prior art and to establish patentable differences. See In re Best, 562 F.2d 1252, 195 USPQ 430 (CCPA 1977) and Ex parte Gray, 10 USPQ 2d 1922 1923 (PTO Bd. Pat.App. & Int.).

Applicant's arguments drawn to the rejection of claims 47, 65-71, 73 and 74 in Paper No. 32 are relevant to the instant rejection.

Applicant argues that claims 47, 65-71, 73 and 74 are not anticipated by Murphy et al because claims 65-71, 73 and 74 depend from claim 47. The argument has been noted but has not been found persuasive for the reasons disclosed above.

11. Claims 47, 65-76 are rejected under 35 U.S.C. § 103 as being unpatentable over Berd et al., Proc. AACR, 1989, 30:382, of record, in view of US Patent No. 5,702,704, No. 5,626,843, No. 5,008,183 or No. 4,232,001 and Geczy et al, of record.

The claims are drawn to a method of treating a malignant tumor in a patient comprising administering to the patient a composition comprising a therapeutically effective amount of tumor cells that are conjugated to a hapten, are of the same tumor type as a malignant tumor of the patient, are autologous to said patient and have been rendered incapable of growing in the body of a human upon injection therein, said composition eliciting at least one of the following upon administration to said patient with an adjuvant: an inflammatory immune response against the tumor; a delayed-type hypersensitivity response against the tumor; and activated T lymphocytes that infiltrate the tumor and repeating said administration at least six times at spaced apart intervals, wherein said tumor cells are selected from a group including melanoma, wherein the treating is useful for treatment of cancer selected from a group including melanoma, wherein said hapten is selected from the group including DNP, wherein the method further comprises administering a therapeutically effective amount of cyclophosphamide prior to administration of said composition, wherein the dose is about 300 mg/M^2, wherein the patient is sensitized with 1-fluoro-2,4-nitrobenzene prior to administration of cyclophosphamide wherein the composition comprises an adjuvant, BGC, wherein the administration prolongs survival of said patient wherein the administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-.

Berd et al teach a method for treating melanoma comprising sensitizing with DNCB, administering 300 mg/M^2 of cyclophosphamide prior to administering a therapeutically effective amount of autologous, irradiated DNP-conjugated melanoma cells mixed with the adjuvant BCG wherein the patients are sensitized

with DNCB prior to cyclophosphamide administration. Berd et al teach as set forth but do not teach a method wherein the vaccine is boosted at least six times at spaced intervals or eliciting at least one of the following upon administration to said patient with an adjuvant: an inflammatory immune response against the tumor; a delayed-type hypersensitivity response against the tumor; and activated T lymphocytes that infiltrate the tumor or wherein the administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4- or that the administration prolongs survival of the patient.

US Patent No. 5,702,704, No. 5,626,843, No. 5,008,183 and No. 4,232,001 and Geczy et al teach as set forth above.

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to combine the methods of Berd et al and the cited patents because it is clearly conventional to repeat antigen administration at least six times at spaced intervals. Although the teaching is drawn to the antibody art, it is well known that T lymphocytes must be stimulated in order to produce the detected antibodies. One of ordinary skill in the art at the time would have expected to successfully use the method with the conventional immunization schedule. Further, although the limitations drawn to an inflammatory immune response against the tumor, a delayed-type hypersensitivity response against the tumor and activated T lymphocytes that infiltrate the tumor, wherein the administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-. are not recited in the reference, the method of the prior art comprises the same method steps as claimed in the instant invention, and the same population, thus the claimed method is anticipated because the method will

inherently lead to an inflammatory immune response against the tumor, a delayed-type hypersensitivity response against the tumor and activated T lymphocytes that infiltrate the tumor wherein the administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-.. See Ex parte Novitski 26 USPQ 1389 (BPAI 1993). It would have been *prima facie* obvious to substitute DNFB for the DNCB of Berd et al because Geczy et al teach that halogenated dinitrobenzenes such a 1-chloro- and 1-fluoro-2,4, dinitrobenzenes are commonly used to elicit delayed hypersensitivity and are clearly closely related haptenic molecules which function to produce the same effects and are therefore functionally equivalent. Finally, as drawn to prolonged survival, the claimed method appears to be the same as that of the prior art method absent a showing of unobvious differences. The office does not have the facilities for examining and comparing applicant's method with the method of the prior art in order to establish that the method of the prior art does not possess the same material structural and functional characteristics of the claimed method. In the absence of evidence to the contrary, the burden is upon the applicant to prove that the method of the prior art does not result in prolonged survival of the patient and is functionally different than the method taught by the prior art and to establish patentable differences. See In re Best, 562 F.2d 1252, 195 USPQ 430 (CCPA 1977) and Ex parte Gray, 10 USPQ 2d 1922 1923 (PTO Bd. Pat.App. & Int.).

12. Claims 43, 44, 47 and 49-76 are rejected under 35 U.S.C. § 103 as being unpatentable over Berd et al., Proc. AACR, 1989, 30:382, of record, in view of US Patent No. 5,702,704, No. 5,626,843, No. 5,008,183 or No. 4,232,001 and Geczy

et al, of record, as applied to claims 47, 65-76 and further in view of Wiseman et al (Western J. Med., 1989, 151:283-288).

The claims are drawn to a composition comprising human tumor cells that are conjugated to a hapten, are of the same tumor type as a malignant tumor of a patient for whom treatment with the composition is intended, are autologous to said patient, have been rendered incapable of growing in the body of a human upon injection therein said composition eliciting an inflammatory immune response against the tumor wherein the tumor is not melanoma, a method for treating a malignant tumor in a human patient comprising administering said composition to the patient wherein said composition elicits, following administration of said composition with an adjuvant, BCG at least one of an inflammatory response against the tumor, a delayed-type hypersensitivity response against the tumor and activated T lymphocytes that infiltrate the tumor, wherein the tumor cells are selected from lung, colon and kidney, wherein said hapten is selected from the group including DNP and TNP, wherein the composition further comprises a carrier which is a saline solution or culture medium, wherein the method is useful for the treatment of lung cancer, colon cancer, or kidney cancer, wherein the method further comprises administering a therapeutically effective amount of cyclophosphamide, 300 mg/M^2, further comprising sensitizing the patient to a therapeutically effective amount of 1-fluoro-2,4, dinitrobenzene prior to administering cyclophosphamide, wherein said administration prolongs survival of the patient, wherein said administration elicits T lymphocytes that infiltrate the tumor said lymphocytes being predominantly CD8+CD4.

Berd et al (1989) record, in view of US Patent No. 5,702,704, No. 5,626,843, No. 5,008,183 or No. 4,232,001, and Geczy et al teach as set forth above but the combined references do not teach a composition or method of treatment wherein the tumor cells are kidney, colon, lung or wherein the cancer treated is kidney cancer, colon cancer or lung cancer.

Wiseman et al teaches compositions comprising autologous irradiated melanoma cancer cells, lung cancer cells, colon cancer cells and kidney cancer cells which are administered to treat patients with melanoma, lung cancer, colon cancer, and kidney cancer (Table 3, page 285), respectively wherein the patients were pretreated with 300 mg/M^2 cyclophosphamide (see abstract - Drug Table) wherein the patients showed increased immunological responses to the cancer (see abstract) wherein patients with, lung cancer, colon cancer, and kidney cancer all showed prolonged survival (Table 3, page 285).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to substitute the lung cancer cells, colon cancer cells or kidney cancer cells of Wiseman et al in the method of Berd et al because each of these cell types had been demonstrated to be immunogenic and to elicit responses in the respective cancer patients and because Berd et al teaches that haptenizing cancer cells increases the efficiency of the immunizing process. One of ordinary skill in the art at the time the invention was made would have been motivated to substitute the tumor cells of Wiseman et al into the method of Berd et al because Wiseman et al teach that their method prolongs survival in some cases and Berd et al teach that their method increases efficiency of the immunization process, thus increased efficiency would be expected to result in increased treatment

efficacy. Further, as drawn to claims 54-55, it is clearly obvious to include a saline carrier in a composition which is prepared for *in vivo* administration because it has been held by the Court that a compound and a carrier are obvious, if it is obvious in the art to utilize a carrier with related compounds. See *In re Rosicky*, 125 USPQ 341 (CCPA 1960). Finally, as drawn to the limitations including, eliciting an inflammatory immune response against the tumor, at least one of an inflammatory response against the tumor, a delayed-type hypersensitivity response against the tumor and activated T, elicits T lymphocytes that infiltrate the tumor said lymphocytes being predominantly CD8+CD4, the claimed compositions and methods of treatment appear to be the same or similar to those of the combined references absent a showing of unobvious differences. The office does not have the facilities for examining and comparing applicant's product or methods with the product or methods of the combined prior art in order to establish that the product or methods of the combined prior art does not possess the same material structural and functional characteristics of the claimed product or methods. In the absence of evidence to the contrary, the burden is upon the applicant to prove that the claimed products and methods are functionally different than those taught by the combined prior art and to establish patentable differences. See In re Best, 562 F.2d 1252, 195 USPQ 430 (CCPA 1977) and Ex parte Gray, 10 USPQ 2d 1922 1923 (PTO Bd. Pat.App. & Int.).

13. Claims 43, 44, 47 and 49-76 are rejected under 35 U.S.C. § 103 as being unpatentable over Berd et al. (1989) of record, in view of US Patent No. 5,702,704, No. 5,626,843, No. 5,008,183 or No. 4,232,001, Geczy et al, of record, as applied

to claims 43, 44, 47 and 49-76 and further in view of Berd et al (Proc. Am. Soc. Clin. Oncol., 1983, Vol 2:56)

The claims are drawn to a composition comprising human tumor cells that are conjugated to a hapten, are of the same tumor type as a malignant tumor of a patient for whom treatment with the composition is intended, are autologous to said patient, have been rendered incapable of growing in the body of a human upon injection therein said composition eliciting an inflammatory immune response against the tumor wherein the tumor is not melanoma, a method for treating a malignant tumor in a human patient comprising administering said composition to the patient wherein said composition elicits, following administration of said composition with an adjuvant, BCG at least one of an inflammatory response against the tumor, a delayed-type hypersensitivity response against the tumor and activated T lymphocytes that infiltrate the tumor, wherein the tumor cells are selected from the group including breast, wherein said hapten is selected from the group including DNP and TNP, wherein the composition further comprises a carrier which is a saline solution or culture medium, wherein the method is useful for the treatment of breast cancer wherein the method further comprises administering a therapeutically effective amount of cyclophosphamide, 300 mg/M^2, further comprising sensitizing the patient to a therapeutically effective amount of 1-fluoro-2,4, dinitrobenzene prior to administering cyclophosphamide, wherein said administration elicits T lymphocytes that infiltrate the tumor said lymphocytes being predominantly CD8+CD4-.

Berd et al., (1989) of record, in view of US Patent No. 5,702,704, No. 5,626,843, No. 5,008,183 or No. 4,232,001, Geczy et al, of record, as applied to

claims 43, 44, 47 and 49-76 teach as set forth above but the combined references do not teach a composition or method of treatment wherein the tumor cells are breast or wherein the cancer treated is breast cancer or wherein the life of the patient is prolonged.

Berd et al (1983) teach a composition and a method for the treatment of breast carcinoma comprising administration of autologous tumor cell/BCG preceded by cyclophosphamide which administration resulted in delayed-type hypersensitivity in 5 patients.

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to substitute the breast cancer cells of Berd et al (1983) in the method of Berd et al (1989) because this cell type had been demonstrated to be immunogenic and to elicit responses in the breast cancer patients and one of ordinary skill in the art would have been motivated to substitute the breast cancer cells of Berd et al (1983) in the method of Berd et al (1989) because Berd et al teaches that haptenizing cancer cells increases the efficiency of the immunizing process, thus increased efficiency would be expected to result in increased treatment efficacy. Further, as drawn to claims 54-55, it is clearly obvious to include a saline carrier in a composition which is prepared for *in vivo* administration because it has been held by the Court that a compound and a carrier are obvious, if it is obvious in the art to utilize a carrier with related compounds. See *In re Rosicky*, 125 USPQ 341 (CCPA 1960). Finally, as drawn to the limitations including, eliciting an inflammatory immune response against the tumor, at least one of an inflammatory response against the tumor, a delayed-type hypersensitivity response against the tumor and activated T, elicits T lymphocytes

that infiltrate the tumor said lymphocytes being predominantly CD8+CD4 and administration leading to prolonged survival of the patient, the claimed compositions and methods of treatment appear to be the same or similar to those of the combined references absent a showing of unobvious differences. The office does not have the facilities for examining and comparing applicant's product or methods with the product or methods of the combined prior art in order to establish that the product or methods of the combined prior art does not possess the same material structural and functional characteristics of the claimed product or methods. In the absence of evidence to the contrary, the burden is upon the applicant to prove that the claimed products and methods are functionally different than those taught by the combined prior art and to establish patentable differences. See In re Best, 562 F.2d 1252, 195 USPQ 430 (CCPA 1977) and Ex parte Gray, 10 USPQ 2d 1922 1923 (PTO Bd. Pat.App. & Int. Finally although the reference does not specifically teach irradiation of the tumor cells prior to administration, it is clear that it would have been *prima facie* obvious and one of ordinary skill in the art would have been motivated to treat the cells to prevent their growth in a body prior to administration, in order to prevent exogenously derived cancer in the patient.

14. Claims 43, 44, 47 and 49-76 are rejected under 35 U.S.C. § 103 as being unpatentable over Berd et al., Proc. AACR, 1989, 30:382, of record, in view of US Patent No. 5,702,704, No. 5,626,843, No. 5,008,183 or No. 4,232,001, Geczy et al, of record, as applied to claims 43, 44, 47 and 49-76 and further in view of Sanda et al (J. Cellular Biochem. Suppl. No. 17, Part D, p. 120) and Moody et al (J. Urol., 1991, 145:293A).

The claims are drawn to a composition comprising human tumor cells that are conjugated to a hapten, are of the same tumor type as a malignant tumor of a patient for whom treatment with the composition is intended, are autologous to said patient, have been rendered incapable of growing in the body of a human upon injection therein said composition eliciting an inflammatory immune response against the tumor wherein the tumor is not melanoma, a method for treating a malignant tumor in a human patient comprising administering said composition to the patient wherein said composition elicits, following administration of said composition with an adjuvant, BCG at least one of an inflammatory response against the tumor, a delayed-type hypersensitivity response against the tumor and activated T lymphocytes that infiltrate the tumor, wherein the tumor cells are prostate, wherein said hapten is selected from the group including DNP and TNP, wherein the composition further comprises a carrier which is a saline solution or culture medium, wherein the method is useful for the treatment prostate cancer, wherein the method further comprises administering a therapeutically effective amount of cyclophosphamide, 300 mg/M^2, further comprising sensitizing the patient to a therapeutically effective amount of 1-fluoro-2,4, dinitrobenzene prior to administering cyclophosphamide, wherein the life of the patient is prolonged, wherein said administration elicits T lymphocytes that infiltrate the tumor said lymphocytes being predominantly CD8+CD4-.

Berd et al. (1989), of record, in view of US Patent No. 5,702,704, No. 5,626,843, No. 5,008,183 or No. 4,232,001, Geczy et al, of record, as applied to claims 43, 44, 47 and 49-76 teach as set forth above but the combined references do not teach a composition or method of treatment wherein the tumor cells are prostate

or wherein the cancer treated is prostate cancer or wherein the life of the patient is prolonged.

Moody et al (J. Urol., 1991, 145:293A) teach that lymphokine-transfected prostate cells generate an anti-tumor effect *in vivo* against rapidly growing rat prostate carcinoma induced by autologous, cells that were not transfected.

Sandra et al addressed the feasibility of gene therapy for human prostate cancer by demonstrating that retroviral vector MFG allows high efficiency transduction of human prostate cancer cells which was demonstrated in primary culture prostate cancer cells from 7 consecutive patients which demonstrates the feasibility of using MFG in genetic therapy for prostate cancer (see abstract).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to substitute the prostate cancer cells of Sandra et al, transfected with lymphokine in the method of Moody et al for the melanoma cells in the method of Berd et al because Moody et al have demonstrated in an appropriate animal model that lymphokine-transfected prostate cells generate an anti-tumor effect *in vivo* against rapidly growing prostate carcinoma and because Sandra et al have demonstrated the feasibility of gene therapy for human prostate cancer by demonstrating the successful transfection of human prostate cancer cells with retroviral vector MFG. One of ordinary skill in the art would have been motivated to substitute the prostate cancer cells of Sandra et al, transfected with lymphokine in the method of Moody et al for the melanoma cells in the method of Berd et al because autologous anti-cancer cell vaccination was known to those of ordinary skill in the art and because Berd et al teach that the method that

haptenizing cancer cells increases the efficiency of the immunizing process, thus increased efficiency would be expected to result in increased treatment efficacy. Further, as drawn to claims 54-55, it is clearly obvious to include a saline carrier in a composition which is prepared for *in vivo* administration because it has been held by the Court that a compound and a carrier are obvious, if it is obvious in the art to utilize a carrier with related compounds. See *In re Rosicky*, 125 USPQ 341 (CCPA 1960). Finally, as drawn to the limitations including, eliciting an inflammatory immune response against the tumor, at least one of an inflammatory response against the tumor, a delayed-type hypersensitivity response against the tumor and activated T, elicits T lymphocytes that infiltrate the tumor said lymphocytes being predominantly CD8+CD4 and administration leading to prolonged survival of the patient, the claimed compositions and methods of treatment appear to be the same or similar to those of the combined references absent a showing of unobvious differences. The office does not have the facilities for examining and comparing applicant's product or methods with the product or methods of the combined prior art in order to establish that the product or methods of the combined prior art does not possess the same material structural and functional characteristics of the claimed product or methods. In the absence of evidence to the contrary, the burden is upon the applicant to prove that the claimed products and methods are functionally different than those taught by the combined prior art and to establish patentable differences. See In re Best, 562 F.2d 1252, 195 USPQ 430 (CCPA 1977) and Ex parte Gray, 10 USPQ 2d 1922 1923 (PTO Bd. Pat.App. & Int. Although neither the Moody et al or Sandra et al reference specifically teaches irradiation of the tumor cells prior to administration, it is clear that it would have been *prima facie* obvious and one of ordinary skill in the art would have been motivated to treat the cells to

prevent their growth in a body prior to administration, in order to prevent exogenously derived cancer in the patient.

15. If applicant disagrees with any rejection set forth in this office action based on examiner's establishment of a priority date of February 28, 1994 for the instantly claimed application serial number 08/203.004, applicant is invited to submit evidence pointing to the serial number, page and line where support can be found establishing an earlier priority date.

16. No claims allowed.

17. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Susan Ungar, PhD whose telephone number is (703) 305-2181. The examiner can normally be reached on Monday through Friday from 7:30am to 4pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Paula Hutzell, can be reached at (703) 308-4310. The fax phone number for this Art Unit is (703) 308-4242.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group receptionist whose telephone number is (703) 308-0196.

Effective, February 7, 1998, the Group and/or Art Unit location of your application in the PTO has changed. To aid in correlating any papers for this application, all further correspondence regarding this application should be directed to Group Art Unit 1642.

SUSAN UNGAR
PATENT EXAMINER

Susan Ungar

April 26, 1999

Exhibit 5

Lab Investigation 62(1): 70A 1990

407 MATURE TERATOMA IN THE LUNG FOLLOWING GONADAL TERATOCARCINOMA.

C.A. Moran and D. Carter, Yale University School of Medicine, New Haven, Ct.

Three cases in which mature teratomas were the only pulmonary manifestation of metastases from gonadal teratocarcinomas - Embryonal carcinoma & Teratoma - were found in the files of the Yale-New Haven Hospital. The patients were all men - 24, 26, and 58 years of age when orchiectomy was performed. The primary tumors each contained a prominent embryonal carcinoma component as well as elements of mature and immature teratoma. The interval from orchiectomy to appearance of pulmonary metastases was less than 4 months and chemotherapy was given prior to removal of the pulmonary metastases in all cases. Two underwent thoracotomy promptly, but the interval was 9 years in the other. In two, the lesions were solitary; the resection at 9 years contained multiple lesions. All were solid and cystic grossly and entirely mature microscopically. Dermal and mucin-secreting glandular epithelium predominated in the cystic areas; fibrous stroma was the predominant feature of the solid areas. Bronchial differentiation, which is usually a component of primary mature and immature teratomas of the lung, was not evident. The course in all cases has been indolent with none of the patients showing manifestations of disease at this time.

408 IMAGE ANALYSIS OF NUCLEOLI AND NUCLEOLAR ORGANIZING REGIONS IN PROSTATIC HYPERPLASIA, PIN, AND PROSTATIC CARCINOMA.

F. Mostofi, I. Sesterhenn, R. Becker, T. Lin, and C. Davis, Armed Forces Institute of Pathology, Washington, DC

Nucleolar orgainizing regions (NORs) are structures on short arms of chromosomes 13, 14, 15, 21, and 22. These are believed to be associated with genes involving rRNA transcription during the late interphase. NORs can be demonstrated in formalin-fixed, paraffin-embedded tissue by use of colloidal silver stains. In normal cells, only one or two of these are visible. NOR counts have been used to differentiate between benign and malignant lesions.

We studied 20 prostates exhibiting areas of hyperplasia, invasive carcinoma (PCa) and intraepithelial neoplasia (PIN). We counted 50 nuclei from each area of hyperplasia, 100 of each PCa area, and 100 from each PIN area. The measurement was carried out using Leitz-TAS system and determined the total combined area of nucleoli and NORs per nucleus. The pooled data of hyperplasia differed from those of PIN and invasive carcinoma. The pooled values of PIN differed only slightly from those of the invasive carcinomas. The determination of NORs is an objective method to differentiate PIN from hyperplasia. The impression, gained on H-E stained sections and immunopathology, that PIN is related to carcinoma is further supported by the NOR values which suggest proliferative activity similar to that of carcinomas.

409 EXPRESSION OF α ACTIN AND DESMIN IN NON-MUSCLE SARCOMAS AND SARCOMA-LIKE LESIONS.

P. Mozzicato, N. Azumi, and K. Leslie, University of Vermont College of Medicine, Burlington, VT and Georgetown University School of Medicine, Washington, DC

β actin is an ubiquitous isotype of this cytoskeletal filament and is not specific for muscle cells. Because many anti-actin antibodies detect this isotype, they are not useful in the diagnosis of sarcomas showing muscle differentiation. The development of monoclonal antibodies specific to muscle isotypes of actin (α and/or γ) and to desmin have proved useful in the diagnosis of leiomyosarcoma and rhabdomyosarcoma. We have found, however, that other mesenchymal lesions without muscle differentiation occasionally express muscle-specific actin. To further define expression of muscle-specific actin among these mesenchymal lesions, we performed immunohistochemical studies using a monoclonal antibody to smooth-muscle-specific isotype of actin (α-SM actin). In addition, monoclonal antibodies to vimentin and to desmin were used. We examined formalin-fixed, paraffin-embedded tissues from cases of malignant fibrous histiocytoma (MFH), nodular fasciitis, and fibrosarcoma. Approximately 25% of the MFH cases and virtually all of the nodular fasciitis cases showed at least focal α-SM actin and, less frequently, desmin positivity. One case of fibrosarcoma showed weak and focal α-SM actin positivity. We conclude that this "spurious" actin and desmin reactivity most likely indicates myofibroblastic differentiation in non-muscle soft tissue lesions. Furthermore, caution should be exercised when diagnosing muscle differentiation in a given mesenchymal lesion based solely on reactivity of these antibodies.

410 * DETECTION OF IMMUNOGLOBULIN (Ig) GENE UTILIZATION BY *IN SITU* HYBRIDIZATION (ISH) WITH OLIGONUCLEOTIDE PROBES

J.D. Mueller, A.A. Long*, K.J. Barrett*, R.S. Schwartz*, H.J. Wolfe, Dept. of Pathology, Laboratory of Molecular Pathology and Div. of Hematology-Oncology, *Dept. of Internal Medicine, Division of Hematology-Oncology Tufts-New England Medical Center, Boston, MA

There is very little information concerning the expressed Ig repertoire of unselected human B lymphocytes. We have developed a ISH method utilizing oligonucleotide probes (23-30 bases long) complimentary to specific mRNA sequences of the constant regions (IgM and IgG) and variable regions of human Ig heavy chain, including each of the 6 defined VH gene families and 2 individual hypervariable regions (CDRs). Probes were labelled to high specific activity ($> 10^9$ cpm/ug) with S35 dATP using terminal deoxynucleotidyl transferase. Cells were prefixed in 2% paraformaldehyde and cytospun onto gelatinized slides. Human B lymphocyte clones producing Ig of known isotype and whose heavy chain nucleic acid sequences were known, were used to develop optimal ISH conditions for each individual probe. Required stringencies were achieved by varying the % formamide of hybridization and wash solutions maintaining constant temperature and salt concentrations. Clones expressing different VH families are readily distinguishable and, under high stringency, clones with sequence homology of up to 87% (2 bases difference in a 23 base sequence) may also be distinguished. In peripheral blood B lymphocytes, the frequency expression of individual VH and CDR sequences by single B cells can be enumerated with this technique, thus permitting a precise determination of the expressed repertoire of human immunoglobulin genes in normal and diseased states.

411 DETECTION OF HUMAN T-CELL LEUKEMIA VIRUS TYPE I DNA IN FORMALIN FIXED PARAFFIN EMBEDDED TISSUE BY POLYMERASE CHAIN REACTION.

K. Mukai, Y. Sato, S. Furuya, T. Kinoshita, and Y. Shimosato, National Cancer Center Research Institute, Tokyo, Japan

Definite diagnosis of adult T-cell leukemia/lymphoma (ATLL) cannot be made in cases which were diagnosed and treated before the development of serological tests for human T-cell leukemia virus type I (HTLV-I). In order to assess whether retrospective study of ATLL is feasible, detection of HTLV-I DNA in formalin fixed paraffin embedded tumor tissue of known ATLL patients was attempted using polymerase chain reaction (PCR). A crude DNA sample from a 5 micron paraffin section was used as a template for in vitro enzymatic amplification of a 120 base long sequence within the gag gene of HTLV-I. In 10 cases of ATLL with positive antibody titers for HTLV-I, the presence of HTLV-1 sequence was detected in all cases. In addition, four of five cases of clinically suspected ATLL but without serological confirmation showed positive reaction for HTLV-I.

The results indicated that PCR is a sensitive tool for detection of HTLV-I DNA in formalin fixed paraffin embedded tissue and that this method can be applied for retrospective study of ATLL.

412 TUMOR INFILTRATING T CELLS IN METASTATIC MELANOMA: INDUCTION BY IMMUNIZATION WITH AUTOLOGOUS, DNP-CONJUGATED TUMOR CELLS

G. F. Murphy, A. Radu, M. Mastrangelo, D. Berd, University of Pennsylvania & Thomas Jefferson University, Philadelphia, PA

The purpose of this study was to determine whether immune mechanisms are responsible for clinically-regressing melanoma metastases in the dermis and subcutis of patients who have been immunized with dinitrophenol (DNP)-conjugated melanoma cells. Two weeks after topical sensitization with dinitrochlorobenzene, subjects pretreated with the immunopotentiating agent cyclophosphamide were injected with vaccine consisting of 10-25X10^6 autologous, irradiated, DNP-conjugated melanoma cells mixed with BCG. A total of 10 excisional biopsies of metastatic nodules from 7 patients were obtained coincident with evidence of clinical regression (erythema, softening, diminution in size). Whereas immune cells could not be detected in tumor nodules prior to therapy, immunohistochemical analysis of regressing lesions after immunization revealed infiltration by preponderantly T cells with cytotoxic/suppressor phenotype. All T cells expressed HLA-DR antigen, and variable numbers showed reactivity for Il-2 receptor and CD45R lymphocyte surface antigen (2H4) associated with suppressor-inducer function. Intercellular adhesion molecule-1 (ICAM-1) was expressed locally by melanoma cells, and the patterns of reactivity coincided precisely with zones of T cell infiltration. Numerous HLA-DR-positive, CD4-positive, Leu 1-negative dendritic cells infiltrated throughout the melanoma nodules. Rare clusters of natural killer cells (Leu 7, Leu 11, and NKH-1-positive) were also detected. In tumors exhibiting focal necrosis, T cells and dendritic cells were no longer prominent, and Leu M1-positive monocyte/macrophages represented the predominant inflammatory cell type.

These data indicate that tumor regression in this model involves activated T cells with potential for cytotoxic function and dendritic cells putatively capable of local antigen presentation. ICAM-1 induction appears to be associated with T cell-tumor cell adhesion induced by this novel mode of immunotherapy.

Exhibit 6

File B

US005702704A

United States Patent [19]

Bucala

[11] Patent Number: 5,702,704

[45] Date of Patent: Dec. 30, 1997

[54] **ANTIBODIES TO IN VIVO ADVANCED GLYCOSYLATION ENDPRODUCTS**

[75] Inventor: **Richard J. Bucala**, New York, N.Y.

[73] Assignee: **The Rockefeller University**, New York, N.Y.

[21] Appl. No.: **486,513**

[22] Filed: **Jun. 7, 1995**

Related U.S. Application Data

[60] Division of Ser. No. 956,849, Oct. 1, 1992, which is a continuation-in-part of Ser. No. 811,579, Dec. 20, 1991, abandoned.

[51] **Int. Cl.**[6] **C07K 16/18**; A61K 39/395

[52] **U.S. Cl.** **424/137.1**; 424/152.1; 424/172.1; 530/387.5; 530/388.2; 530/388.25; 530/389.1; 530/389.3; 530/391.3

[58] **Field of Search** 530/387.5, 388.2, 530/389.25, 389.1, 389.3, 391.3; 424/137.1, 152.1, 172.1

[56] **References Cited**

U.S. PATENT DOCUMENTS

3,654,090	4/1972	Hermans et al. .	
3,850,752	11/1974	Schmurs et al. .	
4,016,043	4/1977	Schmurs et al. .	
4,807,973	2/1989	Goers et al.	424/181.1
5,223,392	6/1993	Cohen .	

FOREIGN PATENT DOCUMENTS

8906798	7/1989	WIPO	G01N 33/53
WO 89/06798	7/1989	WIPO .	

OTHER PUBLICATIONS

Araki et al. J. Biol. Chem. 267:10211–4 (1992).

Horiuchi, S., et al. J. Biol. Chem. 226 (12):7329–7332 (1991).

Nakayama, H., et al. Biochem. Biophys. Res. Com. 162:740–745 (1989).

Suarez, G., et al. J. Biol. Chem. 264 (7):3674–3679 (1989).

Kennedy et al. Diabetologia 26:93–98. (1984).

Vlassara et al., "Functioin of macrophage receptor for nonenzymatically glycosylated proteins is modulated by insulin levels", Diabetes, 35 Supp. 1, p. 13a (1986).

Vlassara et al., "Accumulation of diabetic rat peripheral nerve myeline by macrophages increases with the prescence of advanced glycosylation endproducts", A. J. Exp. Med 160, pp. 197–207 (1984).

Vlassara et al., "Recognition and uptake of human diabetic peripheral nerve myelin by macrophages", Diabetes, 34 No. 6, pp. 553–557 (1985).

Vlassara et al., "High–affinity–receptor–mediated uptake and degradation of glucose–modified proteins: a potential mechanism for the removal of senscent macromolecules", Proc. Natl. Acad. Sci. U.S.A., 82, pp. 5588–5592 (Sep. 1985).

Vlassara et al., "Novel macrophage receptor for glucose–modified proteins is distinct from previously described scavenger receptors", J. Exp. Med. 164, pp. 1301–1309 (1986).

Cerami et al., "Role of nonenzymatic glycosylation in atherogenesis", Journal of Cellular Biochemistry, 30, pp. 111–120 (1986).

Radoff, S. et al., "Characterization of a solubilized cell surface binding protein on macrophages specific for proteins modified nonenzymatically by advanced glycosylation end products", Arch. Biochem. Biophys. 263 No. 2, pp. 418–423 (1988).

Yang, Z. et al., "Two novel rat liver membrane proteins that bind advanced glycosylation endproducts: relationship to macrophage receptor for glucose–modified proteins", J. Exp. Med. 174, pp. 515–524 (1991).

Skolnik, E. et al., "Human and rat mesangial cell receptors for glucose–modified proteins: potential role in kidney tissue remodelling and diabetic nephropathy", J. Exp. Med., 174, pp. 931–939, 1991.

Makita, Z. et al., "Hemoglobin–age: a circulating marker of advanced glycosylation", Science 258, pp. 651–653, 1992.

Horiuchi, S. et al., "Purification of a receptor for formaldehyde–treated serum albumin from rat liver", J. Biol. Chem., 4, 260, pp. 482–488 (1985).

Takata, K. et al., "Scavenger receptor–mediated recognition of maleylated albumin and its relation to subsequent endocytic degradation", Biochem. Biophys. Acta., 984, pp. 273–280 (1989).

Goldstein, J.L. et al., "Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein , producing massive cholesterol deposition." Proc. Nat'l Acad. U.S.A. 76, pp. 333–337 (1979).

Kirstein, M. et al., "Advanced protein glycosylation induces transendothelial human monocyte chemotaxis and secretion of platelet–derived growth factor: role in vascular disease of diabetes and aging", Proc. Nat'l. Acad. Sci. U.S.A., 87, pp. 9010–9014 (1990).

Flückiger, R. et al., "Measurement of nonenzymatic protein glycosylation", Methods Enzymol., 106, pp. 77–87 (1984).

Bucala, R. et al., "Characterization of Antisera to the Addition Product Formed by the Nonenzymatic Reaction of 16 3Hydrozyestrone with Albumin", Mol. Immunol., 20, pp. 1289–1292 (1983).

(List continued on next page.)

Primary Examiner—Christina Y. Chan
Assistant Examiner—Emma Cech
Attorney, Agent, or Firm—Klauber & Jackson

[57] **ABSTRACT**

The circulating advanced glycosylation endproducts Hb-AGE, serum AGE-peptides and urinary AGE-peptides are disclosed as long term markers of diseases and dysfunctions having as a characteristic the presence of a measurable difference in AGE concentration. Diagnostic and therapeutic protocols taking advantage of the characteristics of these AGEs are disclosed. Antibodies which recognize and bind to in vivo-derived advanced glycosylation endproducts are also disclosed. Methods of using these antibodies as well as pharmaceutical compositions are also disclosed, along with numerous diagnostic applications, including methods for the measurement of the presence and amount of advanced glycosylation endproducts in both plants and animals, including humans, as well as in cultivated and systhesized protein material for therapeutic use.

15 Claims, 12 Drawing Sheets

Exhibit 7

Applicant C

US005626843A

United States Patent [19]

Skurkovich et al.

[11] **Patent Number: 5,626,843**

[45] **Date of Patent: May 6, 1997**

[54] **TREATMENT OF AUTOIMMUNE DISEASES, INCLUDING AIDS, BY REMOVEL OF INTERFERONS, TNFS AND RECEPTORS THEREFOR**

[75] Inventors: **Simon V. Skurkovich**, Rockville, Md.; **Boris Skurkovich**, Pawtucket, R.I.

[73] Assignee: **Advanced Biotherapy Concepts, Inc.**, Rockville, Md.

[21] Appl. No.: **25,408**

[22] Filed: **Feb. 26, 1993**

[51] **Int. Cl.**[6] **A61K 39/395**

[52] **U.S. Cl.** **424/140.1**; 604/6

[58] **Field of Search** 424/140.1; 436/547; 604/5, 6

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,362,155 12/1982 Skurkovich .
4,605,394 8/1986 Skurkovich et al. .
4,824,432 4/1989 Skurkovich et al. .
5,231,024 7/1993 Moller et al. .

OTHER PUBLICATIONS

Fahey et al. Clin Exp Immunol., 88: 1–5, 1992.
Skurkovich et al., Medical Hypotheses 41: 177–185, 1993.
Harris et al. Tibtech 11: 42–44, 1993.
Co et al., Nature 351: 501–2, 1991.

Primary Examiner—Toni R. Scheiner
Attorney, Agent, or Firm—Evelyn H. McConathy

[57] **ABSTRACT**

The present disclosure concerns a treatment for autoimmune diseases, including AIDS, by removing interferons, TNFs and receptors therefor, from body fluids. An extracorporeal device exposes fluids from a patient, including blood, plasma, cerelorospinal fluid, and the like, to an immunosorbent to accomplish removal. Following treatment, the fluid is returned to its source.

16 Claims, 1 Drawing Sheet

Exhibit 8

Applicant D

United States Patent [19]

Osther

[11] **Patent Number: 5,008,183**

[45] **Date of Patent: Apr. 16, 1991**

[54] **ASSAY SYSTEM FOR DETECTING ANTIBODY AND A METHOD OF PRODUCING NON-HUMAN IMMUNE ANTIBODY**

[75] Inventor: **Kurt B. Osther, Dallas, Tex.**

[73] Assignee: **Bio-Research Laboratories, Inc., Dallas, Tex.**

[21] Appl. No.: **192,241**

[22] Filed: **May 10, 1988**

[51] Int. Cl.[5] **G01N 33/569**

[52] U.S. Cl. **435/5**; 435/7.32; 436/513; 436/532; 436/547; 436/815; 530/387; 530/830

[58] Field of Search 435/5, 7; 436/513, 532, 436/547, 815; 530/387, 830

[56] References Cited

U.S. PATENT DOCUMENTS

4,132,769	1/1979	Osther	436/543
4,474,877	10/1984	Imagawa et al.	436/513 X
4,487,714	12/1984	Kato et al.	530/391
4,661,445	4/1987	Saxinger et al.	436/528 X
4,725,669	2/1988	Essex et al.	530/395 X
4,774,175	9/1988	Chang et al.	436/811 X

OTHER PUBLICATIONS

Kalheider et al., "Porcine Immunoglobulins. I. Identification of Classes and Preparation of Specific Antisera", J. Immunol. 109(5)992-998 1972.

Thorn et al., "Enzyme Immunoassay Using Novel Recombinant . . . ", J. Clin., Microbiol. 25(7), 1207-1212 (Jul. 1987).

Primary Examiner—Christine Nucker

Attorney, Agent, or Firm—Bryan, Cave, McPheeters & McRoberts

[57] **ABSTRACT**

An improved assay method for detecting the presence of an antibody capable of binding with an antigen of a virus is provided. The improvement comprises using a non-human immune antibody which is reactive with an anti-human antibody as a positive control in the assay. Non-human immune IgM and a method of producing the IgM is also provided.

12 Claims, 1 Drawing Sheet

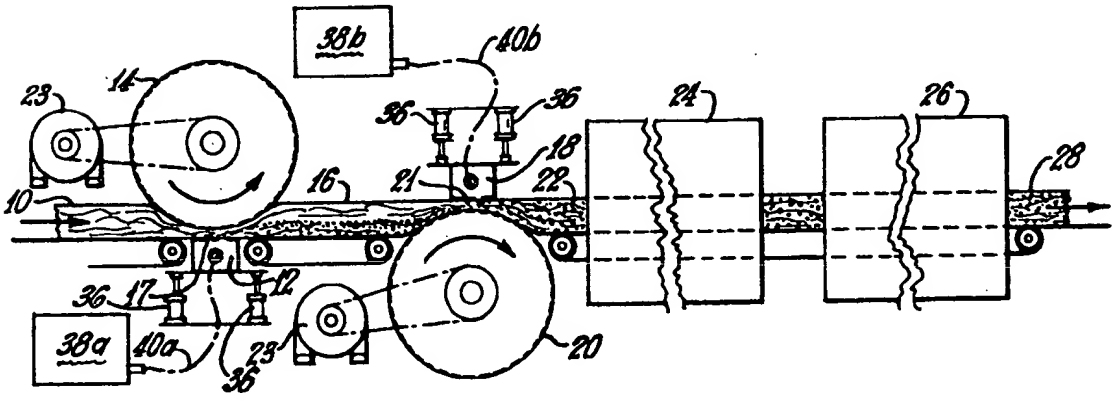


Exhibit 9

Applicant E

United States Patent [19]

Jensen et al.

[11] **4,232,001**

[45] **Nov. 4, 1980**

[54] **METHODS AND MATERIALS FOR DETECTION OF ESTROPHILIN**

[75] Inventors: **Elwood V. Jensen; Eugene R. DeSombre, both of Chicago, Ill.**

[73] Assignee: **University Patents, Inc., Norwalk, Conn.**

[21] Appl. No.: **945,000**

[22] Filed: **Sep. 22, 1978**

[51] **Int. Cl.²** **G01N 33/16; A61K 43/00**

[52] **U.S. Cl.** ..... **424/1; 23/230 B; 260/112 B; 424/12**

[58] **Field of Search** ..... **424/1, 12; 23/230 B; 260/112 B**

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,132,767	1/1979	Tohmatsu et al.	424/1
4,152,410	5/1979	Ishii	424/1
4,160,817	7/1979	Bucouaz et al.	424/1

OTHER PUBLICATIONS

Mercier-Bodard et al., Chem. Abstracts, vol. 89, Sep. 11, 1978, #86004b.

Woods et al., Chem. Abstracts, vol. 88, Feb. 13, 1978 #48714e.

Primary Examiner—Benjamin R. Padgett
Assistant Examiner—Christine M. Nucker
Attorney, Agent, or Firm—Merriam, Marshall & Bicknell

[57] **ABSTRACT**

Antibodies specifically immunologically reactive with estrophilin. Methods and materials for detection and quantification of estrophilin in tissue samples, notably those of human breast cancer tissue.

8 Claims, No Drawings

Exhibit 10

1515

Tumor inflammatory response induced by immunization with autologous melanoma cells conjugated to dinitrophenol(DNP). D. Berd, M.J. Mastrangelo, C. Green, C. Clark, and E. Hart. Thomas Jefferson University, Philadelphia, PA 19107.

Treatment of melanoma patients with an autologous vaccine preceded by low dose cyclophosphamide (CY) induces delayed-type hypersensitivity (DTH) to melanoma cells, and in some cases, regression of metastatic tumors. Now, we are attempting to increase the efficiency of the process by immunizing with tumor cells conjugated to the hapten, DNP. Patients with metastatic melanoma were sensitized to DNP by topical application of dinitrochlorobenzene (DNCB). Two weeks later, they were injected with a vaccine consisting of 10-25x10(6) autologous, irradiated melanoma cells conjugated to DNP and mixed with BCG. CY 300 mg/M^2 IV was given 3 days before DNCB or vaccine. Of 4 patients evaluable so far, 3 have developed a striking inflammatory response in tumor masses after 2 vaccine treatments (8 weeks). Patient #1 developed erythema and swelling in the >50 large (1-3 cm) dermal metastases on her leg and lower abdomen, followed by ulceration and drainage of necrotic material, and some are beginning to regress. Biopsy showed infiltration with CD4+ and CD8+ T lymphocytes. Patient #2 developed erythema and swelling in the skin of her lower abdomen and groin overlying large (8 cm) nodal masses. These have not yet regressed, but have changed in consistency from rock-hard to fluctuant. Patient #3 exhibited moderate erythema in the skin overlying subcutaneous metastases. All 3 patients have developed DTH to both DNCB and to DNP-conjugated autologous lymphocytes. Although these results are preliminary, they suggest that this new strategy may represent a significant advance in the immunotherapy of human melanoma.

1516

Inhibition of Tumor-Induced Suppressor T Lymphocyte (Ts) Activity by Murine Interferon Beta (IFN-B). Deepak M. Sahasrabudhe, University of Rochester Cancer Center, Rochester, NY, 14642

In some tumor models inhibition of Ts-activity is a prerequisite to successful immunotherapy. Based on our data in the DNFB model (J Exp Med 166:1573, 1987) the effect of IFN-B on P815 mastocytoma-induced Ts-activity was evaluated.

In this model, concomitant antitumor immunity (Tc) peaks by Day 10 and is down regulated by Ts by Day 15. Cytotoxicity generated after a mixed lymphocyte tumor culture (MLTC) correlates with in vivo immunity and suppression of cytotoxicity correlates with in vivo Ts-activity.

Tumors were initiated by injecting 2 x 10^6 P815 cells subcutaneously on Day 1. IFN-B (10U, 1000U, 5000U) or buffer were injected i.v. every other day x 5 doses starting on Day 5. On Day 16, MLTC's were set up. Five days later a cytotoxicity assay was performed against ^{51}Cr labelled P815 cells. % specific lysis is shown. Numbers in parenthesis represent the dose of IFN-B.

E:T	Tc Naive	Tc + Ts	Ts	Tc +Ts	Ts (10)	Tc +Ts (10)	Ts (1000)	Tc +Ts (1000)	Ts (5000)	Tc +Ts (5000)
50:1	88	81	0	19	6	22	23	20	81	84
25:1	84	76	0	12	2	21	1	21	63	75
12:1	78	79	2	15	3	24	6	23	58	81
6:1	70	69	1	7	0	9	0	20	38	64
3:1	56	55	0	8	1	13	0	12	21	48

Treatment with IFN-B 5000U every other day x 6 doses abrogated Ts-activity without adversely affecting cytotoxicity. IFN-B may be a useful adjunct in the immunotherapy of selected tumors.

1517

Anti-idiotype monoclonal antibody immunization therapy of cutaneous T cell lymphoma. Chatterjee, M., Foon, K., Seon, B.K., Barcos, M. and Kohler, H., Roswell Park Mem. Inst., Buffalo, NY 14263, and UCSD, San Diego, CA 92161.

Cutaneous T cell lymphoma (CTCL) is an indolent non-Hodgkin's lymphoma which is not cured by standard therapies once it reaches advanced stage. A novel approach to therapy is to use internal image anti-idiotype (Id) mAbs as antigen (Ag) substitute for the induction of immunity. We have generated anti-Id mAb (Ab2) binding to a hybridoma SN2 (Ab1), which recognizes a unique glycoprotein, gp37, expressed by a subset of human leukemic T cells (J. Immunol. 139:1354, 1987). At least 2 of these Ab2 may indeed carry the internal image of the gp37 Ag (J. Immunol. 141:1398, 1988). Recently, we investigated the distribution of gp37 Ag by a sensitive immunoperoxidase staining method using mAb SN2. SN2 had a high specificity for T-leukemia/lymphoma cells and did not react with any normal adult tissues tested including thymus, lymphocytes, bone marrow cells, spleen, liver, kidney, lung, brain, heart, etc. CTCL cells from 5/6 out of 6 patients were strongly positive for gp37 Ag with intense surface membrane staining. The binding of radiolabeled SN2 to CTCL cells was studied for inhibition in the presence of the anti-Id mAbs 4EA2 and 4DC6 which mimic the gp37 Ag. Both clones inhibited the binding 100% and 80% respectively at a concentration of 50 ng. We also generated a murine Ab3 mAb (anti-anti-Id) by immunizing mice with these anti-Id mAb (Ab2). This Ab3 mAb reacts with CTCL cells in an identical fashion as the original Ab1 (SN2). Collectively, these data suggest that Ab2 4EA2 and 4DC6 may be useful for active immunotherapy of CTCL patients. We also plan to study the CTCL patients in a phase I clinical trial to determine the effects of this type of therapy on various components of the immune system (both humoral and cellular) and try to identify the criteria to select patients who may benefit from anti-idiotype vaccine therapy.

1518

Syngeneic murine monoclonal antiidiotypes bearing the internal image of a human breast cancer associated antigen. J. Schmitz and H. Ozer. The Dept. of Microbiology, S.U.N.Y. at Buffalo, Buffalo, NY 14214 and the Division of Medical Oncology, The Univ. of North Carolina at Chapel Hill, Chapel Hill, NC 27599.

According to Jerne's network theory, some antiidiotypes (Ab2) mimic external antigens recognized by specific antibodies (Ab1) and may be used in place of antigen for immunization. The murine monoclonal antibody F36/22 (IgG3, κ), specific for ductal carcinoma antigen (DCA) was used to generate syngeneic monoclonal antiidiotypes bearing the internal image of DCA. Female BALB/c mice were inoculated intraperitoneally every other week with 100 μg of F36/22 coupled to keyhole limpet hemocyanin; the first time in complete Freund's adjuvant and subsequently in incomplete adjuvant. Splenic lymphocytes were fused with the murine cell line P3X63 Ag8.653 3 days after the fourth immunization using 50% polyethylene glycol (m.w. 3000). Two hybrids, MTO-1 and MTO-2, were selected based on the ability of culture supernatants to bind to F36/22 but not to the control antibody 2A31F6 (IgG3, κ) in an enzyme linked immunosorbent assay (ELISA) and cloned by limiting dilution. Paratope specificity of Ab2 was demonstrated in two ELISA assays. First, the binding of labeled F36/22 to DCA was inhibited 100% and 75% by 1.6 μg of MTO-2 and MTO-1 respectively. Second, the binding of labeled Ab2 to Ab1 was inhibited by purified DCA. MTO-1 neither enhances nor inhibits the binding of labeled MTO-2 to Ab1 although in the presence of MTO-2, binding of labeled MTO-1 is enhanced by 100% indicating that these Ab2 recognize distinct idiotopes. Rabbits immunized bi-weekly with MTO-1 or MTO-2 developed antibodies that bound specifically to DCA demonstrating that MTO-1 and MTO-2 bear the internal image of DCA. These data suggest that MTO-1 and MTO-2 could potentially be utilized to immunize high risk patients against progression or development of DCA positive tumors.

Exhibit 11

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Immunology, 1970, 19, 189.

1766
7011

Lymphocyte Transformation in Contact Sensitivity

A. F. Geczy and A. Baumgarten

School of Pathology, University of New South Wales, Sydney, Australia

(*Received* 3rd *March* 1969)

Summary. Leucocyte suspensions containing 97–100 per cent lymphocytes were prepared from the peripheral blood of unsensitized guinea-pigs as well as animals previously sensitized with 1-chloro-2,4-dinitrobenzene. The lymphocytes were dinitrophenylated and cultured for 5 days *in vitro*. Transformed cells (8–22 per cent) and an increased (100–1000 fold) uptake of tritiated thymidine were observed only in cultures of lymphocytes from sensitized animals. Smaller numbers of transformed cells (1–2 per cent) were found in only a few cultures of sensitive lymphocytes incubated with either dinitrophenylated autologous erythrocytes or serum proteins. These results strongly suggest that transformation may specifically follow the conjugation of DNFB with live lymphocytes from sensitized donors.

INTRODUCTION

Halogenated dinitrobenzenes such as 1-chloro- and 1-fluoro-2,4-dinitrobenzene (DNCB and DNFB respectively) are commonly used to elicit delayed hypersensitivity (Bloom and Chase, 1967; Turk, 1967). The dermal reactions evoked by these substances in sensitized animals may result from an interaction between specifically sensitive cells and dinitrophenylated acellular skin components which act as antigens (Eisen and Tabachnick, 1958). However, it is possible that the highly reactive halogenated dinitrobenzenes (Eisen, Orris and Belman, 1952) may also react directly with lymphocytes, and subsequently initiate the dermal reaction by releasing substances such as the macrophage inhibiting factor (David, Al-Askari, Lawrence and Thomas, 1964; Bloom and Bennett, 1966) and the lymph node permeability factor of Willoughby, Boughton, Spector and Schild (1962). Since specific antigens can induce lymphocyte transformation *in vitro* Pearmain, Lycette and Fitzgerald, 1963; Elves, Roath and Israëls, 1963), a method is available for the determination of the ability of DNFB to act as a specific antigen by inducing transformation of sensitized, living lymphocytes. The occurrence of transformation of dinitrophenylated lymphocytes *in vitro* would suggest that DNFB can act directly on lymphocytes *in vivo*.

Furthermore, if delayed reactions are carrier-specific (Gell and Wolstencroft, 1967) the complete antigen which induces transformation of dinitrophenylated lymphocytes should contain a moiety supplied by the lymphocyte. It seems unlikely that a moiety with an identical molecular configuration can be supplied by acellular constituents of the skin. For the same reasons, it seems necessary to suppose that the antigen which initially induces hypersensitivity is also derived from dinitrophenylated lymphocytes formed during the sensitizing exposure to the chemical. An acquisition of information in this way would support a mechanism of peripheral sensitization suggested by Medawar (1958) and Strober and Gowans (1965). This paper reports the *in vitro* transformation of living, dinitrophenylated lymphocytes.

MATERIALS AND METHODS

Preparation of DNFB solutions

For conjugation with lymphocytes, erythrocytes and serum proteins, a stock solution of DNFB (Fluka A. G., Buchs, S. G.) was prepared by dissolving approximately 300 μg DNFB per ml in 0·2 M (pH 8·5) phosphate buffer at 60°. The actual concentration of DNFB in the buffer was determined spectrophotometrically.

For conjugation with bovine serum albumin (BSA; Commonwealth Serum Laboratories, Melbourne, Vic.) a DNFB solution was prepared in 0·2 M citrate–phosphate–borate buffer (Teorell and Stenhagen, 1938) at the appropriate pH.

Preparation of DNCB solutions

For sensitization, a stock solution of DNCB (British Drug Houses, England) in distilled water was prepared by dissolving approximately 300 μg DNCB per ml and the concentration of DNCB then adjusted to 40 μg/ml under spectrophotometric control. For testing, a stock solution of DNCB was prepared by dissolving a known quantity of DNCB in olive oil B.P. (N.S.W. Government Stores). The stock solution was diluted as required with a 1 : 9 mixture of olive oil and petroleum ether (B.P. 60°–80°, A.R. grade, B.D.H.).

Protein determinations were done according to the method of Lowry, Rosebrough, Farr and Randall (1951), using BSA as the standard.

Sensitization and skin testing

Outbred albino female guinea-pigs (600–800 g) were sensitized and tested according to Baumgarten and Wilhelm (1969). Briefly, the animals were sensitized by multiple injections of 40 μg DNCB in the nuchal skin. Eleven to 14 days after sensitization, skin testing was done by applying 20 μl DNCB solution (0·03, 0·1, 0·3 and 1·0 per cent) to each of eight randomly distributed sites on the closely shaved dorsal skin of the trunk using Hamilton 250 μl syringes (New Jersey, U.S.A.) fitted with blunted 26 G hypodermic needles. The solution was spread with a rounded glass rod in the direction of hair growth over a 1·5–2·0 cm^2 area. The lesions were examined after 20–24 hours for the presence of erythema; the animals were subsequently given intravenous Evans blue (1·2 ml/kg body weight, as a solution containing 25 mg dye per ml of 0·45 per cent saline) to demonstrate the development of increased vascular permeability (Voisin and Toullet, 1960).

Preparation of lymphocytes

Blood was usually obtained from guinea-pigs by cardiac puncture, occasionally by severing the jugular vein. It was placed in sterile bottles (containing 10–15 glass beads 3–5 mm diameter), defibrinated by gentle shaking for 5–10 minutes and transferred into fresh bottles. To each millilitre of blood was added 0·3 ml of 1 per cent methyl cellulose (U.S.P. Grade, Dow Chemicals Co.) solution in 0·2 M phosphate buffer, pH 7·2. After mixing by inversion, the blood was allowed to sediment for 45 minutes at 37°. The uppermost two-thirds of the top (leucocyte-rich) layer was removed and centrifuged at 400 *g* for 5 minutes. The supernatant was then separated and the cells washed twice with Hanks's solution (C.S.L., Melbourne, Vic.). After further processing as described below, the cells were suspended in a culture medium (Medium 199; C.S.L., Melbourne, Vic., with 20–40 per cent guinea-pig serum).

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Heat-killed lymphocytes were prepared by incubating 1 ml suspension containing 0·6–8 × 10^6 lymphocytes in Hanks's solution at 56° for 30 minutes. Cell death was ascertained by the eosin technique, i.e. by incubating the cells for 5–10 minutes in 0·5 per cent solution of Eosin B (Matheson, Coleman and Bell, New Jersey, U.S.A.) in saline, and counting a total of 200–300 eosin-stained and unstained cells from each preparation (Hanks and Wallace, 1958).

Preparation of peritoneal cells

Four guinea-pigs were injected intraperitoneally with 20 ml Hanks's solution and then killed. The peritoneal cavities were opened and the fluid was aspirated and centrifuged at 200 *g* for 10 minutes. The pellet of cells from each animal was washed twice with Hanks's solution and then suspended in culture medium.

Preparation of blood macrophages

Cardiac blood from four guinea-pigs was sedimented with 'Methocel' as described above. The upper two-thirds of the leucocyte-enriched layer were then discarded, while the lower third was centrifuged at 200 *g* for 10 minutes. The cells in the resulting pellet were washed twice with Hanks's solution and suspended in culture medium.

Cell counts

Total white cell counts were done in Neubauer counting chambers using 2 per cent aqueous acetic acid as diluent. Differential counts were performed on smears prepared from pellets obtained by centrifuging the cell suspension and stained with May–Grünwald-Giemsa.

The percentage of transformed cells was determined by counting at least 300 cells from each preparation using the longitudinal method of Dacie and Lewis (1963).

Conjugation with DNFB

(a) *Bovine serum albumin* (BSA). Two cuvettes (in the same light path) were placed in the reference and two in the test compartment of a Unicam S.P. 800 B spectrophotometer. One cuvette in each compartment contained BSA (20 mg/ml) and the other DNFB (20 μg/ml), both in buffer at the appropriate pH. After equilibration for 5 minutes the solutions in the test compartment were mixed to give an effective concentration of 10 mg/ml and 10 μg/ml for BSA and DNFB, respectively; the cuvettes were then replaced. The reaction was followed for 72 hours at 325 mμ, since it had been previously found that at this wavelength there was a maximal difference between the absorbance of BSA and DNFB and the DNFB–BSA conjugate. The pH of the reaction mixture was then lowered to about 3 with 2N hydrochloric acid. An equivalent volume of distilled water was added to the reference cells to obtain a corresponding dilution of DNFB and BSA. The optical density of the reaction mixture was then again determined. This procedure was adopted to measure any absorbance due to a coloured compound (presumably 2,4-dinitrophenol; DNP) which slowly formed on standing in alkaline DNFB solutions.

(b) *Lymphocytes.* In preliminary experiments 10^6 twice washed leucocytes were incubated with 0–100 μg/ml concentrations of DNFB in phosphate buffer pH 8·5 for periods up to 10 hours. In subsequent experiments the lymphocyte pellet was reconstituted in a solution of DNFB (10 μg/ml) in the phosphate buffer. The volume of the DNFB solution was adjusted to give a final concentration of about 10^6 cells/ml. After 30 minutes incubation, the

suspension was reduced to pH approximately 6·3 by gassing with carbon dioxide, centrifuged at 200 *g* for 5 minutes, the supernatant removed, the cells washed thrice with Hanks's solution and then suspended in culture medium. Control cultures of unconjugated lymphocytes were treated in a similar fashion, except that the cells were not incubated with DNFB.

(c) *Erythrocytes.* Erythrocytes obtained from the residual defibrinated and sedimented blood by centrifugation for 10 minutes at 200 *g* were washed twice with Hanks's solution and conjugated with DNFB as described above for lymphocytes.

(d) *Serum proteins.* Autologous serum proteins were conjugated with DNFB by mixing equal portions of serum (obtained from centrifuged blood) with DNFB solution (containing 10 μg DNFB per ml) and incubating the mixture at 37° for 30 minutes.

Preparation of mixed cultures

Unconjugated live lymphocytes ($0·6–1·3 \times 10^6$) were mixed with either an approximately equal number of autologous: (1) conjugated heat-killed lymphocytes, (2) unconjugated heat-killed lymphocytes, (3) conjugated erythrocytes, or with 2 ml autologous conjugated serum protein solution. A fifth set of mixed cultures was prepared by mixing $0·6–0·8 \times 10^6$ conjugated live lymphocytes with a similar number of autologous unconjugated heat-killed lymphocytes.

Carbon studies

A carbon suspension was prepared by diluting 'Pelikan' ink (Batch No. C11/1431a Gunther Wagner Werke, West Germany) in proportion 1 : 5 with Hanks's solution. Approximately 0·05 ml of the carbon suspension was added to: (1), 3 ml of each peritoneal cell preparation (10^6 cells/ml); (2), 1 ml of each blood macrophage preparation (10^6 leucocytes/ml); (3), 1 ml of each lymphocyte preparation ($0·5 \times 10^6$ lymphocytes/ml). The cells were incubated with carbon at 37° for 2 hours, then centrifuged and washed 3 or 4 times with Hanks's solution. After the last centrifugation the cells were spread on microscope slides and stained with May-Grünwald-Giemsa.

Uptake of tritiated thymidine

Thymidine-6-T(n) (Radiochemical Centre, England) was used in amounts of 2 μCi per 10^6 lymphocytes. In the first experiment duplicate cultures of conjugated live lymphocytes from two sensitized and two unsensitized guinea-pigs were incubated with thymidine for 5 days. Each day the cultures were sampled and the uptake of thymidine was determined. In the second experiment portions of 3-day duplicate cultures of conjugated lymphocytes from two sensitized guinea-pigs were pulsed with thymidine for 1, 2, 4 or 6 hours.

In the third experiment duplicate cultures of conjugated lymphocytes were prepared from four sensitized guinea-pigs. Portions of 3-day cultures were pulsed with thymidine for 2 hours. The remainder of each culture was incubated for a further 2 days and the percentage of transformed cells then determined. In the fourth experiment duplicate cultures were prepared of both conjugated and unconjugated lymphocytes from twelve sensitized and twelve unsensitized guinea-pigs. From a further seven sensitized animals were established: (1), seven mixed cultures of unconjugated live lymphocytes and autologous conjugated erythrocytes; (2), five cultures of conjugated live lymphocytes; (3), five mixed cultures of unconjugated live lymphocytes and autologous conjugated serum proteins; (4), four mixed cultures of unconjugated live lymphocytes and autologous conjugated

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FIG. 1. The effect
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heat-killed lymphocytes. All cultures were pulsed with thymidine for 2 hours on the 3rd day of incubation. After incubation for the stated periods, the cells were washed thrice with Hanks's solution and then prepared for liquid scintillation counting (Tri-Carb, Packard) according to the method of Mahin and Lofberg (1967).

Autoradiography

Cultures of conjugated lymphocytes were prepared from the cardiac blood of four sensitized guinea-pigs and unconjugated lymphocytes from the blood of two unsensitized animals. Subsequently, $0{\cdot}5\times10^6$ unconjugated lymphocytes were incubated with 2 μCi tritiated thymidine for 16 hours. Portions containing approximately $0{\cdot}5\times10^6$ conjugated lymphocytes from cultures aged 0, 3 or 5 days were also incubated with 2 μCi tritiated thymidine for 16 hours. After incubation the cells were washed thrice with Hanks's solution, centrifuged and spread on microscope slides. The slides were coated with NTB2 autoradiographic emulsion (Eastman-Kodak, U.S.A.), exposed for 5 days, developed and then stained with May-Grünwald-Giemsa.

RESULTS

CONJUGATION OF 1-FLUORO-2,4-DINITROBENZENE (DNFB) WITH BOVINE SERUM ALBUMIN (BSA)

The conjugation of DNFB with protein is known to occur rapidly at alkaline pH values (Eisen, Orris and Belman, 1952). The extent of conjugation of DNFB with protein in the range suitable for use with living cells *in vitro* was ascertained using BSA. BSA was chosen for convenience, because light scattering of cell suspensions rendered them unsuitable for direct spectrophotometric measurement of DNFB conjugation. The reaction rates of BSA with DNFB at different pH values are illustrated in Fig. 1. It is noteworthy that appreciable conjugation occurs even at pH 5·5.

During this investigation a yellow colour, probably due to 2,4-dinitrophenol, was found to develop gradually in alkaline DNFB solutions standing for several weeks. This colour

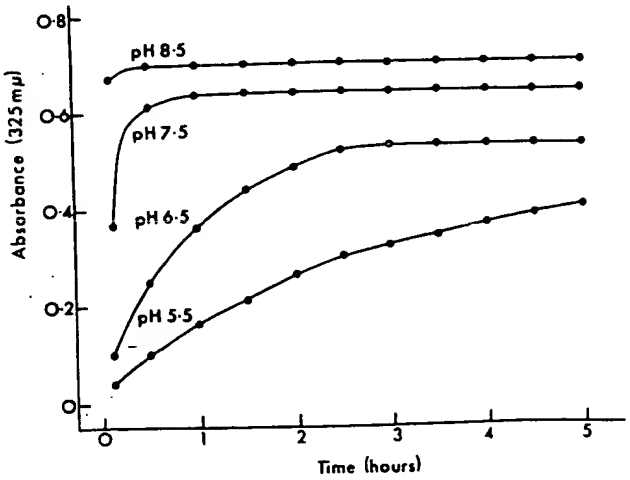

FIG. 1. The effect of pH on the rate of conjugation of 1-fluoro-2,4-dinitrobenzene with bovine serum albumin.

disappeared at about pH 3, unlike that of DNFB–albumin conjugates. Accordingly, the absorbance of the reaction mixtures was measured after 72 hours both at the reaction pH (see Fig. 1) and at pH 3. After correction for dilution by acid, absorbance was similar at both pH values, suggesting that the formation of the yellow colour was not sufficiently rapid to interfere appreciably with the measurements recorded in Fig. 1. In view of the results with BSA (Fig. 1) conjugation of DNFB with living cells was carried out at pH 8·5 for 30 minutes.

CONJUGATION OF DNFB WITH LEUCOCYTES

Preliminary experiments showed that preparations of 10 μg DNFB per 10^6 leucocytes contained no more eosin-stained (non-viable) leucocytes than control cultures containing no DNFB. A concentration of 10 μg DNFB per 10^6 cells was therefore selected for subsequent conjugation. A preparation of 10^6 leucocytes and the same number of erythrocytes contained approximately 3·3 mg protein.

DIFFERENTIAL COUNT

Differential counts of leucocyte suspensions immediately prior to culture showed that virtually all (97–100 per cent) cells were lymphocytes, and the remainder granulocytes and occasional monocytes. The suspensions contained approximately equal numbers of red and white cells.

TOTAL CELL COUNT

The total cell count decreased by up to 30 per cent by the 3rd day of culture but usually diminished no further in the subsequent 2 days (Table 1). Two possible exceptions were the counts of conjugated lymphocytes and lymphocytes incubated with conjugated erythrocytes (both from sensitized donors) which, on the 5th day, fell to about 50 per cent of

TABLE 1

EFFECT OF INCUBATION ON CELL COUNTS IN CULTURE

	No. of animals	Serum	No. of cells per ml (× 10^6) 0 days	3 days	5 days
Conjugated lymphocytes					
unsensitized donors	14§	A	1·2–2·0 (1·6)	1·1–1·4 (1·3)	1·1–1·5 (1·3)
	2	H*	1·9 (1·9)	1·6, 1·7 (1·65)	1·6 (1·6)
sensitized donors	25‡§	A	0·8–2·0 (1·3)	0·7–1·4 (0·9)	0·3–1·1 (0·4)
	2	H†	1·8, 2·0 (1·9)	1·6, 1·7 (1·65)	1·7, 1·8 (1·75)
Conjugated red cells					
sensitized donors	17‡§	A	0·8–1·3 (0·9)	0·8–1·1 (0·9)	0·4–0·6 (0·5)
Conjugated serum proteins					
sensitized donors	17‡§	A	0·8–1·3 (1·0)	0·6–1·2 (0·9)	0·7–0·9 (0·8)
Unconjugated lymphocytes					
unsensitized donors	2	H†	2·0 (2·0)	1·5, 1·7 (1·6)	1·4, 1·7 (1·55)
	4	A	1·1–2·0 (1·5)	0·9–1·6 (1·2)	1·3–1·8 (1·5)
sensitized donors	14	A	1·1–1·8 (1·3)	1·0–1·5 (0·9)	0·9–1·4 (1·1)
	4	H*	1·2–1·8 (1·5)	1·0–1·6 (1·2)	1·2–1·4 (1·3)

A: autologous. H: homoglogous.
* Serum from sensitized donors. † Serum from unsensitized donors. ‡ Includes the eleven guinea-pigs whose lymphocytes were also incubated with dinitrophenylated red cells and serum proteins. § Cultures from these animals were prepared in duplicate. Values in parentheses represent means.

their values on the 3rd day (Table 1). This relatively pronounced decrease in the number of lymphocytes possibly indicates *in vitro* lysis of sensitive cells exposed to DNFB or to dinitrophenylated erythrocytes. A similar decrease was not observed in lymphocyte cultures from sensitized donors incubated with dinitrophenylated serum proteins. In mixed cultures of autologous live and heat-killed lymphocytes, either of which was conjugated with DNFB, the total cells decreased by the 5th day to about one-third the initial number. This decrease corresponded to that observed in cultures of only live lymphocytes (Table 1).

VIABLE CELL COUNT

On the day of collection (0 day) 77–100 per cent of all cells were viable; on the 3rd and 5th day of culture, the viability decreased to 30–80 per cent and 10–75 per cent respectively. The percentage of viable cells varied widely from culture to culture (Table 2) and no special trends of either decreased or increased viability could be discerned. In mixed cultures of live and autologous heat-killed lymphocytes, the decrease in the percentage of viable cells (from 40 to 60 per cent initially to 20–40 per cent on the 3rd or 5th day) was proportional to that in cultures containing only live lymphocytes.

TABLE 2

EFFECT OF CULTURE ON VIABLE CELL COUNT

	No. of animals	Serum	Per cent viable cells		
			0 days	3 days	5 days
Conjugated lymphocytes unsensitized donors	14§	A	95–100 (96)	58–71 (61)	39–58 (43)
	2	H*	96, 97 (96·5)	52, 60 (56)	18, 41 (30)
sensitized donors	25‡§	A	85–100 (92)	36–80 (60)	10–58 (41)
	2	H†	95, 100 (97·5)	52, 58 (55)	19, 24 (27)
Conjugated red cells sensitized donors	17‡§	A	79–100 (91)	33–80 (71)	28–60 (53)
Conjugated serum proteins sensitized donors	17‡§	A	77–100 (93)	32–70 (59)	26–60 (47)
Unconjugated lymphocytes unsensitized donors	4	A	95–100 (97)	58–80 (67)	20–75 (50)
	2	H†	95, 100 (98)	60 (60)	31, 48 (40)
sensitized donors	14	A	91–100 (93)	58–65 (60)	38–50 (43)
	4	H*	92–98 (96)	45–60 (52)	30–68 (40)

A: autologous. H: homoglogous.
* Serum from sensitized donors. † Serum from unsensitized donors. ‡ Includes the eleven guinea-pigs whose lymphocytes were also incubated with dinitrophenylated red cells and serum proteins. § Cultures from these animals were prepared in duplicate. Values in parentheses represent means.

LYMPHOCYTE TRANSFORMATION

Only a small number (<2 per cent) of transformed cells was seen in mixed cultures of unconjugated live lymphocytes and either autologous conjugated serum proteins (in two of seventeen cultures) or autologous conjugated erythrocytes (in six of seventeen cultures). By contrast, 8–22 per cent transformed cells (Fig. 12) was seen in 5-day cultures of live lymphocytes conjugated with DNFB (Table 3). Similarly, 5–10 per cent transformed cells were present in 5-day mixed cultures of conjugated live lymphocytes and autologous unconjugated heat-killed lymphocytes; the proportion of transformed cells would have been even higher (10–20 per cent) if allowance had been made for the presence of about 50 per cent non-viable lymphocytes in the initial inoculum.

TABLE 3

EFFECT OF CONJUGATION ON TRANSPORMATION OF CULTURED LYMPHOCYTES

	No. of animals	Serum	Per cent transformed cells	
			3 days	5 days
Conjugated lymphocytes				
unsensitized donors	14§	A	0	0
	2	H*	0	0
sensitized donors	25‡§	A	0	8–22 (14)
	2	H†	0	8, 16 (12)
Conjugated red cells				
sensitized donors	17‡§	A	0	0–1 (<1)
Conjugated serum proteins				
sensitized donors	17‡§	A	0	0–2 (<1)
Unconjugated lymphocytes				
unsensitized donors	4	A	0	0
	2	H†	0	0
sensitized donors	14	A	0	0
	4	H*	0	0

A: autologous. H: homoglogous.
* Serum from sensitized donors. † Serum from unsensitized donors. ‡ Includes the eleven guinea-pigs whose lymphocytes were also incubated with dinitrophenylated red cells and serum proteins. § Cultures from these animals were prepared in duplicate. Values in parentheses represent means.

CARBON UPTAKE

Carbon-laden cells were observed both in preparations of blood macrophages and peritoneal cell cultures aged 0, 3 or 5 days. By contrast, no cells which phagocytosed carbon were detected in 0-, 3- or 5-day cultures of conjugated live lymphocytes from sensitized donors.

UPTAKE OF TRITIATED THYMIDINE

In an initial experiment conjugated lymphocytes from two unsensitized guinea-pigs incorporated relatively little radioactivity (<2500 counts/min per 10^6 lymphocytes) in the first 4 days of incubation with tritiated thymidine but took up rather more radioactivity on the 5th day (about 18,000 counts/min per 10^6 lymphocytes). By contrast, conjugated lymphocytes from two sensitized animals incorporated respectively about 25,000 counts min and 55,000 counts/min per 10^6 lymphocytes on the 3rd and 5th day of incubation.

TABLE 4

LACK OF CORRELATION BETWEEN UPTAKE OF TRITIATED THYMIDINE AND TRANSFORMATION IN CULTURES OF CONJUGATED LYMPHOYCTES

	Sensitized donors							
	1		2		3		4	
	a	b	a	b	a	b	a	b
Radioactivity in 3-day cultures (counts/min/10^3 cells)	135	132	135	134	83	64	90	74
Transformed cells in 5-day cultures (per cent)	16	9	11	12	10	9	10	7

a, b: duplicate cultures.

TABLE 5

EFFECT OF DONOR SENSITIVITY ON UPTAKE OF TRITIATED THYMIDINE BY CULTURED LYMPHOCYTES

	No. of animals	Radioactivity (counts/min per 10^6 live lymphocytes)
ensitized donors		
Conjugated red cells	7[a]	110–880 (480)
Conjugated serum proteins	5[a]	96–700 (360)
Conjugated killed lymphocytes	4[a]	580–830 (696)
Conjugated live lymphocytes	17[a,b]	15,000–132,500 (73,000)
Unconjugated live lymphocytes	12[b]	38–300 (73)
Unsensitized donors		
Conjugated live lymphocytes	12[c]	38–500 (88)
Unconjugated live lymphocytes	12[c]	34–550 (110)

The superscripts a, b and c designate different batches of animals.

In the second experiment 3-day cultures of conjugated lymphocytes from two sensitized guinea-pigs incorporated 20–30 times more thymidine than similar cultures of unconjugated lymphocytes. Furthermore, conjugated lymphocytes pulsed with thymidine for 2 hours took up almost as much radioactivity (29,000 counts/min per 10^6 lymphocytes) as lymphocytes pulsed for 6 hours (32,000 counts/min per 10^6 lymphocytes).

In the third experiment, duplicate cultures of conjugated lymphocytes from four sensitized animals exhibited an increased uptake of thymidine on the 3rd day of incubation and the presence of transformed cells on the 5th day. However, the uptake of radioactivity appeared not to vary with the percentage of transformed cells (Table 4).

The results of the fourth experiment have been recorded in Table 5. Tritiated thymidine was incorporated in relatively low amounts (<880 counts/min per 10^6 lymphocytes) in cultures of: (1) conjugated lymphocytes from unsensitized donors, (2) unconjugated lymphocytes from either sensitized or unsensitized donors, (3) mixed cultures of unconjugated live lymphocytes from sensitized donors and autologous conjugated (a) erythrocytes, (b) serum proteins, or (c) heat-killed lymphocytes. By contrast, the uptake of radioactivity exceeded 15,000 counts/min per 10^6 lymphocytes in all cultures of conjugated live lymphocytes from sensitized guinea-pigs.

AUTORADIOGRAPHY

Preparations of freshly collected unconjugated lymphocytes from unsensitized guinea-pigs contained no cells which became labelled after 16 hours incubation with tritiated thymidine. By contrast, in 16-hour cultures of conjugated lymphocytes from sensitized donors, 6 (0·34 per cent) of 1750 examined leucocytes were labelled. The labelled cells resembled large lymphocytes (Figs. 2–4, cf. Fig. 5). In 3-day cultures, 48 (6·9 per cent) of 700 examined leucocytes were labelled. The labelled cells varied from ones resembling large lymphocytes to others which approached the appearance of transformed cells (Figs. 6-8, cf. Fig. 9). They also varied in the degree of labelling (Figs. 6–9). Two cells which exhibited markedly different labelling were united by a cytoplasmic strand (Fig. 10), probably depicting the last stage in cellular division. In 5-day cultures, 120 (18·2 per cent) of 660 examined leucocytes were labelled. However, only 58 (8·8 per cent) of these were 'typical' transformed cells (Fig. 11, cf. Fig. 12), the other 62 (9·4 per cent) appearing to represent intermediate stages between a large lymphocyte and a transformed cell. The

FIG. 3.

FIG. 2.

FIG. 4.

FIG. 5. LY cortisol/DNFB + incub'd w/ tritiated thymidine for 16 hrs — the cells exhibit scanty cytoplasm + variable degree of labelling; unlabelled cells are illus'd in FIG 5. ×2200.

FIG. 6.

FIG. 7.

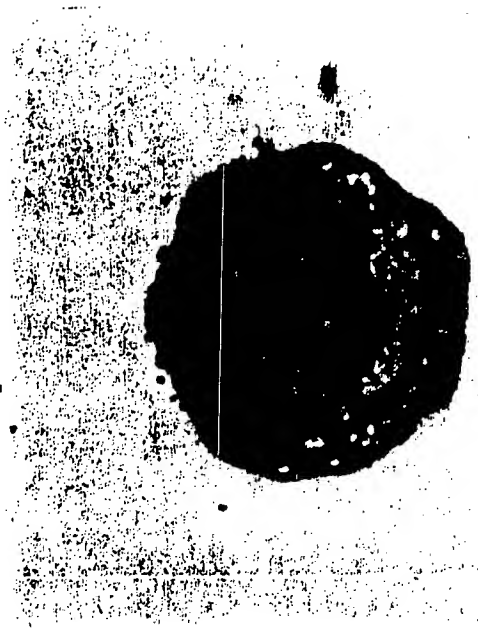

FIG. 8.

FIG. 9.

FIGS. 6–9. Cells from a 3-day-old culture of dinitrophenylated lymphocytes, illustrating variation in size and degree of labelling after 16 hours incubation with tritiated thymidine; an unlabelled cell from the same culture is shown in Fig. 9. × 2200.

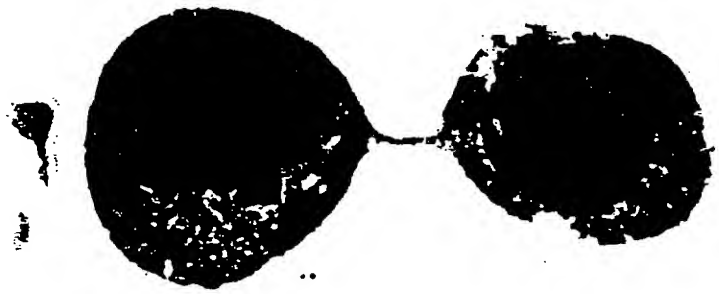

FIG. 10. Cells from a 3-day-old culture of dinitrophenylated lymphocytes. The cells are joined by a strand of cytoplasm. ×2200.

FIG. 11. A labelled, transformed cell from a culture aged 5 days. ×2200.

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FIG. 12. A transformed cell from a culture aged 5 days, stained with May-Grünwald-Giemsa. × 2200.

degree of labelling varied considerably in different cells but did not seem to be related to the microscopic appearance of the cells. Nevertheless, the majority of transformed cells (62·4 per cent) were labelled.

DISCUSSION

The transformation of lymphocytes *in vitro* in the presence of specific antigens has been amply documented (Pearmain, Lycette and Fitzgerald, 1963; Elves, Roath and Israëls, 1963; Coulsen and Chalmers, 1967). However, despite the frequent use of DNCB in investigations of delayed hypersensitivity (Landsteiner and Jacobs, 1935; Turk, 1967; Bloom and Chase, 1967), no studies appear to have been made of its effect on cultured lymphocytes. The use of DNFB in this work was prompted by its ability to form stable conjugates with protein (Eisen, Orris and Belman, 1952).

In defining the conditions for the use of DNFB the extent of its conjugation with BSA has been investigated in the pH range similar to which lymphocytes can be exposed.

It may be assumed that after 4 hours at pH 8·5, no further reaction occurs between DNFB and BSA since there is virtually no additional increase in optical density after 30 minutes. Therefore, it seems reasonable to assume that the reaction between DNFB and protein in the cell suspensions is also approximately complete in 30 minutes. However, the availability of cell protein for conjugation may be limited, on one hand, by the adherence of serum protein to the cells (despite washing), and, on the other hand, by the intracellular localization of the bulk of the protein. It seems unlikely that free DNFB can enter the cells in view of its high reactivity at the usually alkaline pH of the extracellular fluid, and the availability of protein for conjugation in both the adsorbed serum

and the cell membrane. However, if DNFB does enter the cell, it can presumably react with intracellular protein since some conjugation with albumin is found even at pH 5·5.

In cultures of conjugated cells from the sensitized guinea-pigs, the total number of cells decreases by the 5th day to about one-third of its initial value. Since a similar fall does not usually occur in other cultures, the conjugated cells from sensitized donors probably undergo lysis of immune origin. Moreover, since the total number of cells similarly decreases in cultures of unconjugated heat-killed cells used with conjugated live cells, as well as unconjugated live cells used with conjugated heat-killed cells, dinitrophenylated lymphocyte antigen can possibly induce lysis of unconjugated living lymphocytes. By contrast, it appears that transformation can be induced only in cells directly exposed to DNFB.

The transformation of conjugated lymphocytes from sensitized donors has been established both on a morphological basis and by the incorporation of tritiated thymidine. It appears to be a specific reaction of conjugated, sensitive lymphocytes, since neither transformed cells nor appreciable thymidine uptake are observed in unconjugated cell cultures from sensitized animals or conjugated and unconjugated cell cultures from unsensitized donors. The transformation appears to be a specific outcome of the exposure of sensitized lymphocytes to DNFB, since little or no transformation occurs when live lymphocytes are incubated with either conjugated, heat-killed lymphocytes, erythrocytes or serum proteins.

In some cultures containing conjugated erythrocytes or serum proteins, the presence of a small number of transformed cells can be interpreted as a low degree of responsiveness to these antigens. It is also possible, however, that a small amount of residual unconjugated DNFB remains adsorbed to erythrocytes or is present in serum and later reacts with lymphocytes.

Conjugation of DNFB with lymphocytes therefore appears to be a necessary prerequisite to transformation. The high reactivity of DNFB suggests that it mainly combines with protein adsorbed to the cell or a component of the cell membrane. Since the adsorbed protein is probably derived from the serum, and conjugated serum proteins do not induce appreciable transformation, it seems likely that the union resulting in transformation occurs at the cell membrane. Transformation of lymphocytes from sensitized animals following *in vitro* conjugation with DNFB suggests that a similar mechanism may operate *in vivo*.

Migrating lymphocytes may initially react with the chemical in the skin and induce hypersensitivity after undergoing changes either locally or in the regional lymph nodes. Sensitive lymphocytes may subsequently react with freshly applied chemical and having been stimulated by the specific antigen, release factors which elicit the local reaction.

ACKNOWLEDGMENTS

We wish to thank Dr P. R. L. C. Lam-Po-Tang, Haematologist, Prince of Wales Hospital and Mr D. Paix, Physicist, Prince Henry Hospital, Sydney for advice on the preparation of lymphocyte cultures and specimens for liquid scintillation counting respectively. This work was supported by a grant and Fellowship (to A.B.) from the Asthma Foundation of New South Wales.

BAUMGARTEN, A. and WILHE
permeability responses ir
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317.
BLOOM, B. R. and BENNETT,
reaction *in vitro* associatec
sensitivity.' *Science*, 153, 8(
BLOOM, B. R. and CHASE, M
delayed-type hypersensit
151.
COULSON, A. S. and CHALME
of human blood lymphoc
tissue culture.' *Immunology*,
DACIE, J. V. and LEWIS, S. M
tology, 3rd edn, p. 55. Chu
DAVID, J. R., AL-ASKARI, S
THOMAS, L. (1964). 'D
vitro.' *J. Immunol.*, 93, 264
EISEN, H. N., ORRIS, L. and I
tion of delayed allergic sk
the dependence of elicitati
with protein.' *J. exp. Med.*
EISEN, H. N. and TABACHNI
of allergic contact dermati
ELVES, M. W., ROATH, S. anc
'The response of lymphocy
vitro.' *Lancet*, i, 806.
GELL, P. G. H. and WOLS
'Antigenic specificity.' *Brit.*
HANKS, J. H. and WALLACE,
tion of cell viability.' *Proc.*
188.

REFERENCES

Baumgarten, A. and Wilhelm, D. L. (1969). 'Vascular permeability responses in hypersensitivity. II. The reaction to 2,4-dinitrochlorobenzene.' *Pathology*, **1**, 317.

Bloom, B. R. and Bennett, B. (1966). 'Mechanism of a reaction *in vitro* associated with delayed-type hypersensitivity.' *Science*, **153**, 80.

Bloom, B. R. and Chase, M. W. (1967). 'Transfer of delayed-type hypersensitivity.' *Progr. Allergy*, **10**, 151.

Coulson, A. S. and Chalmers, D. G. (1967). 'Response of human blood lymphocytes to tuberculin P.P.D. in tissue culture.' *Immunology*, **12**, 417.

Dacie, J. V. and Lewis, S. M. (1963). *Practical Haematology*, 3rd edn, p. 55. Churchill, London.

David, J. R., Al-Askari, S., Lawrence, H. S. and Thomas, L. (1964). 'Delayed hypersensitivity *in vitro*.' *J. Immunol.*, **93**, 264.

Eisen, H. N., Orris, L. and Belman, S. (1952). 'Elicitation of delayed allergic skin reactions with haptens: the dependence of elicitation on hapten combination with protein.' *J. exp. Med.*, **95**, 473.

Eisen, H. N. and Tabachnick, M. (1958). 'Elicitation of allergic contact dermatitis.' *J. exp. Med.*, **108**, 773.

Elves, M. W., Roath, S. and Israëls, M. C. G. (1963). 'The response of lymphocytes to antigen challenge *in vitro*.' *Lancet*, **i**, 806.

Gell, P. G. H. and Wolstencroft, R. A. (1967). 'Antigenic specificity.' *Brit. med. Bull.*, **23**, 21.

Hanks, J. H. and Wallace, J. H. (1958). 'Determination of cell viability.' *Proc. Soc. exp. Biol. (N.Y.)*, **98**, 188.

Landsteiner, K. and Jacobs, J. (1935). 'Studies on the sensitization of animals with simple chemical compounds.' *J. exp. Med.*, **61**, 643.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). 'Protein measurement with the Folin phenol reagent.' *J. biol. Chem.*, **193**, 265.

Mahin, D. T. and Lofberg, R. T. (1966). 'A simplified method of sample preparation for determination of tritium, carbon-14, or sulphur-35 in blood or tissue by liquid scintillation counting.' *Analyt. Biochem.*, **16**, 500.

Medawar, P. B. (1958). 'The homograft reaction.' *Proc. roy. Soc. B*, **149**, 145.

Pearmain, G., Lycette, R. R. and Fitzgerald, P. H. (1963). 'Tuberculin-induced mitosis in peripheral blood leucocytes.' *Lancet*, **i**, 637.

Strober, S. and Gowans, J. L. (1965). 'The role of lymphocytes in the sensitization of rats to renal homografts.' *J. exp. Med.*, **122**, 347.

Turk, J. L. (1967). *Delayed Hypersensitivity*, p. 31. North-Holland, Amsterdam.

Teorell, T. and Stenhagen, E. (1938). 'Ein Universalpuffer für den pH-bereich 2,0 bis 12,0.' *Biochem. Z.*, **299**, 416.

Voisin, G. A. and Toullet, F. (1960). 'Modifications of capillary permeability in immunological reactions mediated through cells.' *Ciba Foundation Symposium on Cellular Aspects of Immunity*, p. 373. J. & A. Churchill, London.

Willoughby, D. A., Boughton, B., Spector, W. G. and Schild, H. O. (1962). 'A vascular permeability factor isolated from normal and sensitized guinea-pig lymph node cells.' *Life Sci.*, **1**, 347.

Exhibit 12

Applicant ✓

Clinical Investigation

Clinical Responses With Active Specific Intralymphatic Immunotherapy for Cancer—A Phase I-II Trial

CHARLES L. WISEMAN, MD; V. SRINAVASA RAO, PhD, PharmD; PETER S. KENNEDY, MD; CARY A. PRESANT, MD; J. DOUGLAS SMITH, MD; *and* ROBERT J. McKENNA, MD, *Los Angeles*

We evaluated the method of active specific intralymphatic immunization to treat cancer in 32 patients with various tumor types as part of a broad-based phase I-II evaluation and describe the results of 3 sequential series. In series 1, the patients (n = 13) received 2 or more injections of autologous, cryopreserved, irradiated tumor cells directly into the lymphatic system through the cannulation of a dorsal pedal lymphatic channel. In series 2, the patients (n = 7) received low-dose cyclophosphamide, 300 mg per m², 3 days before the autologous cell vaccine was administered. Series 3 (12 patients) was similar to series 2 except that the tumor cells were treated with cholesteryl hemisuccinate immediately before irradiation. Patients received from 2 to 6 injections of cells, depending on availability, at 2-week intervals. In all, 91 treatments are evaluated in this study. Clinical responses occurred in 7 of the 32 patients and were seen in all 3 series with about the same frequency. These responses occurred in cases of melanoma, lung cancer, colon cancer, and sarcoma. Regressions occurred in both visceral and subcutaneous sites. There was little toxicity, the chief side effect being local discomfort or inflammation. This experience indicates that active specific intralymphatic immunotherapy is safe, produces antitumor effects, and requires more investigation to increase the frequency and duration of observable tumor regression.

(Wiseman CL, Rao VS, Kennedy PS, et al: Clinical responses with active specific intralymphatic immunotherapy for cancer—A phase I-II trial. West J Med 1989 Sep; 151:283-288)

The historical enthusiasm for using autologous tumor cells as immunogens has resulted in a number of clinical trials, and some positive results warrant further attention.[1] We have been interested in several recent developments that encourage further investigation of such an approach. We began a study to investigate and extend the original report of Juillard and co-workers, who described regression of metastatic tumors after inoculating irradiated tumor cells directly into the lymph nodes through the dorsal pedal lymphatic vessels.[2]

Direct intralymphatic immunization is an attractive concept for a number of theoretic reasons. Such a technique could circumvent possible immunosuppressive mechanisms of a primary tumor. Regional lymph nodes vary widely in immunologic responsiveness.[3] Some studies indicate that nodes close to a primary tumor have decreased functional competence and increased suppressor-cell activity as compared with nodes distant from the primary tumor.[4]

The intralymphatic approach has been studied in animals[5,6] with positive results and in several human tumor categories. In addition to the work of Juillard and colleagues, augmented immunologic responses were reported in breast cancer and in renal cancer by Adler and associates, although the clinical significance of these responses remains open.[7,8]

Our initial findings suggested that the method reproducibly elicited substantial biologic effects, with a significant elevation of the fraction (and absolute number) of circulating CD4+ lymphocytes. We observed that this elevation occurred after each immunization and in almost every patient.[9,10] In a few melanoma patients, two subpopulations of CD4+ were identified.[11]

Since the initiation of this study, we have made several modifications based on possibly useful newer techniques. The reports by Berd and Mastrangelo indicated that the use of low doses of cyclophosphamide may selectively block suppressor-cell functional activity, thereby leading to augmented specific immune responses.[12] After our initial experience, we introduced this as a pretreatment for our patients (series 2 and 3). We then changed the program in an attempt to increase potential cell-surface immunogenicity of tumor cells by reducing membrane-lipid microviscosity. We introduced the methods of Skornick and associates, using a brief incubation with cholesteryl hemisuccinate (series 3).[13]

Patients and Methods

Tumor Vaccine

Surgically removed tumor was dispersed by collagenase and deoxyribonuclease according to methods previously de-

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scribed.[9] The cells were frozen slowly using a programmed cryogenic freezer (Union Carbide, Indianapolis) and stored over liquid nitrogen. On demand, the cells were thawed rapidly and washed in Hanks' medium. Adherent cells were removed by incubating at 37°C, and the remaining population was then irradiated to 200 gray from a cobalt 57 source. Dosimetry and supervision of the radiation procedure were provided by Armand Bouzaglou, MD, and John Sevilla, MD. For patients in series 3, vaccine preparation was modified. After thawing and washing, the cells were suspended in a polyvinyl pyrolidone-Hanks' solution, pH 7.2, containing 250 μg per ml cholesteryl hemisuccinate (Sigma), adjusted to 1×10^6 viable cells per milliliter, and incubated three to four hours at room temperature, washed, and resuspended in lactated Ringer's solution 5 to 10×10^6 viable cells per milliliter before irradiation. The irradiated cells were suspended in a lactated Ringer's solution at a concentration of 10 to 15 $\times 10^6$ viable cells per milliliter. Viability was assessed by trypan blue exclusion. Aliquots of the initial preparation and the irradiated vaccine were routinely tested for bacterial contamination by culture as recommended by federal protocol.[14] The viability of the tumor cells was generally about 70% or higher, although occasionally a patient was treated with a vaccine of lower viability.

Patients

All patients provided signed informed consent before enrolling in this program. Approval and periodic review had been provided by the Institutional Review Board of the St Vincent Medical Center (Los Angeles), both initially and after the sequential modifications of the program. All patients received a complete medical history and physical examination, complete blood counts, biochemical profile, and such x-ray films, computed tomograms, and isotope scans as needed to evaluate the extent of metastatic involvement and the dimensions of at least one measurable indicator lesion. Therapy was not initiated until at least three weeks had elapsed from previous chemotherapy, radiation therapy, or surgical procedure requiring general anesthesia. No additional antitumor therapy was permitted for any patient during the period of treatment on this protocol.

Autologous irradiated tumor cells, suspended at a concentration of 10 to 15×10^6 viable cells per milliliter, were injected over several minutes into a dorsal pedal lymphatic

TABLE 1.—*Clinical Characteristics of 32 Patients Receiving Active Specific Intralymphatic Immunotherapy*

Patient	Age, yr	Sex	Primary Cancer	ECOG Scale of Metastases*	Site(s) of Metastases	Previous Chemotherapy Regimens, No.
Series 1						
1	58	♂	Melanoma	1	Lung	0
2	45	♂	Melanoma	1	Lung, liver	0
3	76	♂	Melanoma	1	Nodes	3
4	28	♂	Melanoma	4	Skin	3
5	74	♂	Melanoma	4	Lung, liver	0
6	44	♂	Colon	4	Nodes, ascites, liver	1
7	28	♂	Colon	2	Liver	0
8	75	♀	Colon	2	Liver	1
9	54	♂	Lung	2	Lung	0
10	35	♂	Lung	1	Lung	0
11	41	♂	Nasopharynx	4	Liver, bone, lung	3
12	64	♂	Renal	1	Lung, chest wall	0
13	61	♂	Renal	2	Kidney	0
Series 2						
14	61	♂	Colon	2	Lung, abdomen	1
15	48	♂	Colon	1	Liver	0
16	32	♂	Melanoma	3	Liver, lung	0
17	48	♀	Melanoma	1	Skin	2
18	42	♀	Melanoma	2	Abdomen, nodes	1
19	48	♂	Renal	2	Lung	0
20	41	♂	Unknown	3	Abdomen, lung	1
Series 3						
21	64	♀	Colon	2	Liver, bone	1
22	62	♀	Lung	4	Skin, lung, kidney	1
23	78	♂	Lung	3	Lung, liver	0
24	68	♂	Melanoma	2	Abdomen	1
25	45	♂	Melanoma	2	Abdomen, nodes	3
26	56	♂	Melanoma	3	Scalp, liver, nodes	3
27	38	♀	Melanoma	2	Lung, nodes	0
28	32	♂	Melanoma	1	Lung	3
29	62	♂	Melanoma	1	Lung, skin	1
30	45	♀	Melanoma	3	Lung	2
31	65	♂	Renal	4	Bone, lung	3
32	45	♀	Sarcoma	2	Skin, liver	1

ECOG—Eastern Cooperative Oncology Group

*The ECOG scale measures the performance status of a patient using a scale of 0 (fully active, able to carry on all predisease activities without restrictions) to 4 (completely disabled, totally confined to bed or chair).

channel isolated after Evans blue was infiltrated and lidocaine hydrochloride was administered for local anesthesia according to methods used for lymphangiography. All cutdown procedures were done by the staff of the Department of Radiology, St Vincent Medical Center. Patients received the vaccine at two- to four-week intervals as supplies permitted. Unless required for other medical considerations, admission to hospital was not needed. For those patients receiving cyclophosphamide (Cytoxan, Bristol-Myers Oncology Division), the drug was injected intravenously three days before vaccine inoculation at a dose of 300 mg per m^2 following pretreatment with the intramuscular administration of triethylperazine, 10 to 15 mg (Torecan, Roxane Laboratories, Inc).

TABLE 2.—*Distribution of Tumor Types Per Series*

Tumor Types	Patients, No. Series 1	Series 2	Series 3	Total
Melanoma	5	3	7	15
Colon cancer	3	2	1	6
Lung cancer	2	0	2	4
Renal cancer	2	1	1	4
Miscellaneous*	1	1	1	3
Total	13	7	12	32

*Nasopharyngeal 1, adenocarcinoma of unknown primary 1, atrial myxosarcoma 1

Results

The roster of patients, together with clinical characteristics, is provided in Table 1. As mentioned, our initial experience, series 1, represents the tumor vaccine program unmodified. Series 2 and 3 differ in that patients were treated with low-dose cyclophosphamide. Series 3 comprises those patients whose vaccine underwent pretreatment with cholesteryl hemisuccinate; additionally, series 3 patients, similar to the patients in series 2, received a priming, immunomodulating dose of cyclophosphamide before the vaccine treatment. There were 13 patients in series 1, 7 in series 2, and 12 in series 3. The program was initiated June 1, 1981; we evaluated responses and survival as of July 15, 1988. Table 2 shows the tumor types and their distribution according to series.

All patients had advanced cancer, usually with pulmonary or intra-abdominal metastases. The median age was 48 years (range, 26 to 78). There were 8 women. Eleven patients had had no previous therapy; an occasional patient had had extensive previous treatment. The median performance score on the Eastern Cooperative Oncology Group scale was 2; six had scores of 4 (completely bedridden) and five had

TABLE 3.—*Therapy and Responses*

Patient	Primary Cancer	Viable Cells/Cycle, × 10^6/ml	Tumor Response	Time to Progression, wk	Survival, wk	Comment
Series 1						
1	Melanoma	5.0, 25.0, 50.0, 56.0	Mixed	12.0	48.1	Partial remission, pulmonary; CNS failure
2	Melanoma	20.0, 15.0	Progression	4.0	13.0	
3	Melanoma	10.0, 14.0, 20.0	Progression	10.0	146.7	
4	Melanoma	20.0, 40.0	Progression	9.1	9.1	
5	Melanoma	10.0, 5.0, 35.0, 60.0, 20.0	Progression	12.9	13.1	
6	Colon	3.0, 6.0	Mixed	4.0	7.7	Progression of Virchow's node
7	Colon	6.0, 4.5, 4.8	Stable	19.7	24.0	
8	Colon	6.0, 9.0, 33.0	Stable	32.4	43.3	
9	Lung	10.0, 20.0, 12.0	Progression	8.0	10.1	
10	Lung	10.0, 2.0	Complete remission	22.7	43.1	See text
11	Nasopharynx	14.0, 8.0, 1.5	Progression	7.7	14.4	
12	Renal	20.0, 10.0, 30.0	Progression	10.0	200.4	
13	Renal	14.0, 14.0, 17.0	Progression	6.0	6.0	
Series 2						
14	Colon	10.0, 12.0, 10.0	Mixed	14.6	73.9	See text
15	Colon	11.0, 14.0, 14.0	Progression	6.3	169.4	Active, slowly progressive disease
16	Melanoma	20.0, 18.0	Progression	3.6	37.7	
17	Melanoma	18.0, 21.0, 12.5	Complete remission	67.9	79.0	See text
18	Melanoma	24.0, 28.0, 30.0	Stable	13.4	21.9	
19	Renal	12.0, 12.0, 13.0, 3.0, 7.0, 6.0	Progression	6.9	45.7	
20	Unknown	6.0, 9.0	Progression	7.0	9.9	
Series 3						
21	Colon	11.0, 6.0	Progression	7.0	15.6	
22	Lung	10.0, 9.7	Progression	4.6	8.1	
23	Lung	9.0, 9.0	Progression	7.0	19.9	
24	Melanoma	13.0, 9.0, 9.0	Progression	19.0	57.7	
25	Melanoma	10.0, 10.0, 10.0	Progression	10.0	69.3	
26	Melanoma	10.0, 12.0	Progression	5.7	6.4	
27	Melanoma	6.0, 8.0	Progression	12.4	17.1	
28	Melanoma	13.0, 13.0, 12.0, 10.0	Progression	9.0	39.7	
29	Melanoma	10.0, 10.0, 11.0	Partial remission	25.1	69.7	
30	Melanoma	8.0, 9.0, 9.0	Progression	6.9	13.1	
31	Renal	17.0, 18.0	Progression	4.4	11.9	
32	Sarcoma	11.0, 12.0, 14.0	Mixed	5.3	42.9	See text

CNS=central nervous system

scores of 3—that is, about a third of the patients were partially or completely bedridden.

Table 3 depicts the number of cycles of immunization per patient, the amount of viable cells of each vaccine treatment, the maximum response according to standard criteria of the Southwestern Oncology Group, the time to progression, and the duration of survival from the initiation of treatment. For 32 patients in the study, there were a total of 91 treatments. Examples of objective responses are depicted in Figures 1, 2, and 3. Figure 1 shows a chest x-ray film of patient 10, a 35-year-old man who underwent an exploratory thoracotomy for operable but not resectable, large-cell undifferentiated lung cancer. The patient received two vaccine treatments, with the subsequent disappearance of the lung nodule and improvement of mediastinal widening, both on chest x-ray film and computed tomography (CT). The patient was then treated by his referring physician with chemotherapy and irradiation to consolidate the remission, but the disease recurred five months later.

Figure 2 shows the computed tomograms of patient 29, a 62-year-old man who had an excisional biopsy of pulmonary nodules diagnostic of metastatic melanoma. In addition to pulmonary metastases, the patient had noticed a 1-cm nodule in the right thigh shortly before the initiation of vaccine treatment. The pulmonary disease showed regression, the thigh mass disappeared, and the patient had a reduction of serum concentrations to the monoclonal antibody CA 125 from 91 to 33 ng per ml eight weeks following the start of treatment. The patient then showed progressive disease in the lung and also brain metastases after six months.

Figure 3 shows CT scans taken of a 45-year-old woman (patient 32) who presented with an atrial myxoma invading the great vessels. After surgical resection she was treated with infusional chemotherapy with doxorubicin (Adriamycin, Adria Laboratories) hydrochloride, but hepatic metastases developed. She received three cycles of autologous irradiated tumor cell vaccine as per the methods described for series 3 patients. The follow-up scans five weeks later showed disappearance of several vaguely demarcated liver lesions and reduction of a major lesion from 4 by 4 cm to 1.5 by 1.5 cm. New lesions were identified on the skin and also in the lungs, but the hepatic lesions regressed.

Patient 1 had multiple vaguely defined pulmonary infiltrates, which showed more than 50% regression simultaneous with the development of brain metastases. Patient 14 had an exploratory laparotomy with partial resection of a retroperitoneal mass, not identifiable on computed tomography. Signs of a bowel obstruction developed 14 weeks later, again without tumor visible by CT scan. At an operation the patient had tumor obstructing the distal small bowel but the surgeon saw that the original tumor mass was more than 50% reduced.

Patient 17 had a resection of one of several large breast masses palpable and visible on mammography. After three vaccine treatments, the mass adjacent to the resected tumor appeared to enlarge and was resected. Interestingly, only necrotic debris was obtained. When the patient relapsed 68 weeks later, there was a large, palpable, and darkly pigmented nodule in the region from which a biopsy was previously taken, as well as several other areas in both breasts.

The median survival for all patients was 36 weeks. We note that occasionally a patient had an unusually long survival. Patient 12, with recurrent renal carcinoma in the chest wall, showed a greater than 25% increase in the size of the tumor; he was subsequently treated with a regimen of interferon, without significant benefit, then radiation therapy. Lung metastases also developed. Nonetheless, he remains ambulatory and reasonably active 299 weeks after the autologous intralymphatic vaccine therapy started. Patient 15 had progressive liver metastases on a CT scan after three treatments with autologous irradiated tumor cells and was treated with two courses of intrahepatic chemotherapy with mito-

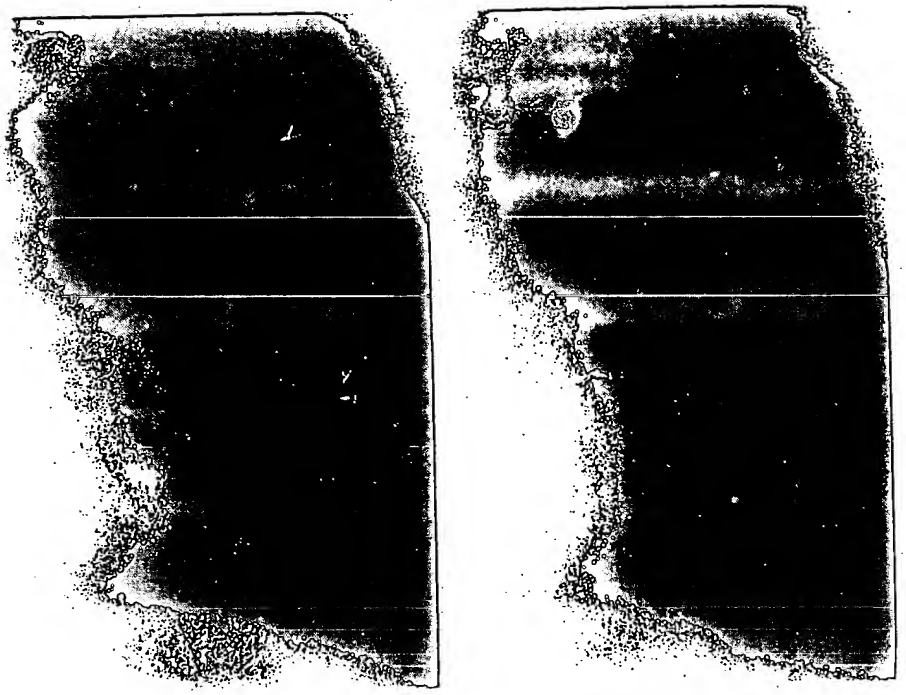

Figure 1.—Left, A chest x-ray film of patient 10 before vaccine therapy shows a lesion (arrow) in the left hilum. Right, After 2 cycles, the left hilar lesion has disappeared.

mycin and fluorouracil. He has declined further medical evaluation but continues to work more than 169 weeks since treatment was initiated.

Direct toxicity was limited to an occasional local infection, rarely fever or other systemic reactions. There were no instances of regional adenopathy, nor did any patient have symptoms of immediate hypersensitivity. There was no evidence of tumor enhancement or acceleration of the clinical course. Wound infections and difficult cannulations were seen primarily in series 1 patients (three patients each). Fever was identified briefly in one patient, who received antipyretics and had a normal temperature after 24 hours. One patient (number 8) with colon carcinoma and extensive pelvic tumor had a self-limited deep vein thrombophlebitis in the contralateral leg; this condition required admission to hospital and anticoagulation but resolved without sequelae. The patient had no further complications until her demise from progressive distant metastases 32 weeks later. Another patient (number 13) with renal carcinoma died suddenly at home; at autopsy a pulmonary embolus was identified. This event is not unexpected in this population, but there is a possibility that the cutdown procedure, requiring immobilization on a gurney for 60 to 120 minutes, contributed to its development. Nonetheless, patients in the later series were not similarly affected.

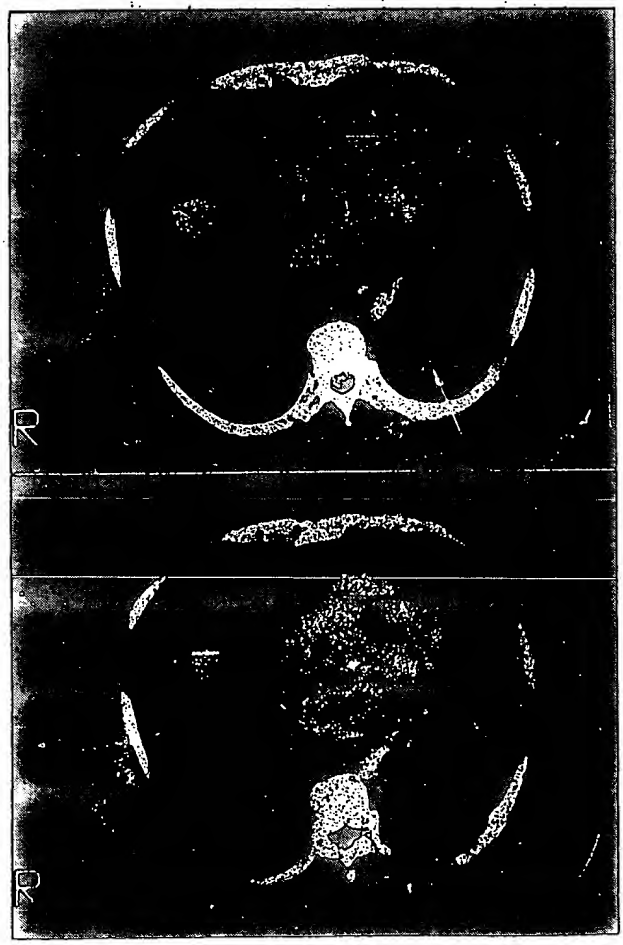

Figure 2.—Top, A computed tomogram at the start of treatment in patient 29 shows a large pulmonary nodule (arrow) in the left posterior lung. Bottom, A computed tomogram taken 60 days later shows regression of the nodule in the left posterior lung.

Discussion

This report confirms the clinical interest of active specific immunotherapy using autologous cells through intralymphatic injection. The original report by Juillard and colleagues in 1978 showed that tumor vaccines could elicit regressions of metastatic disease,[2] a controversial notion at a time when it was widely held that immunologic mechanisms would be ineffective except for subclinical, micrometastatic disease.[1] We describe here objective regressions in 7 of 32 patients studied. It should be noted that these very ill patients all had tumors for which effective or even palliative therapy is marginal. The responses have been limited and incomplete, however, and the problem of mixed responses is disappointing. McCune and co-workers, in a study of renal carcinoma, interpret mixed responses as a strong argument for the polyclonality of tumors.[15] The response of patient 32 is encouraging, even if there was growth of tumor at other sites, given the severity of liver involvement and the rarity of regressions under these circumstances.

In evaluating the population for possible prognostic factors, we note that all of the responding patients were younger than 65 and most had performance status scores of 2 or less. The mean age of the seven responders, however, was 50, and that age was not appreciably different from that of the group as a whole (mean age, 51.3 years).

Previous chemotherapy was not a contraindication to a response, contrary to the experience of Weisenburger and colleagues.[16] That study, albeit involving allogeneic tissue

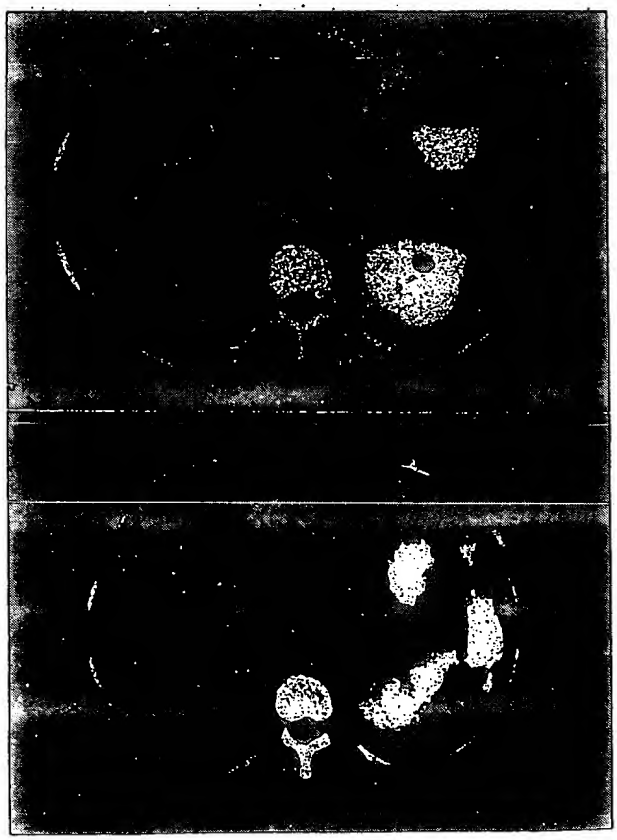

Figure 3.—Top, A computed tomogram taken of patient 32 shows an atrial myxoma (arrow) invading major vessels. Bottom, 70 days later, there is regression of the tumor nodule in the anterior lobe of the liver, with regeneration of liver tissue locally and in the posterior area of the right lobe.

culture cells, identified responses in 9 of 34 patients when intralymphatic therapy was the first method used but no responses in 13 patients who had received previous chemotherapy.[16] In contrast, five of our seven responders had previous chemotherapy.

Although we describe an evolving strategy for implementing active specific intralymphatic immunotherapy, the role of cyclophosphamide and of pretreatment of the tumor cells with cholesteryl hemisuccinate is still unclear. Skornick and associates reported responses in 7 of 21 patients treated with cholesteryl-modified autologous tumor cells.[17] This process did not amplify the response rate in our study. The incidence of clinical responses is not meaningfully different among the three series: 3 of 13 (23%) in series 1, 2 of 7 (29%) in series 2, and 2 of 12 (17%) in series 3.

The method and experience described suggest the existence of host responses that can have potentially useful effects against disseminated malignancy. Attempts to identify the existence of relevant serum antibodies, described by Ahn and co-workers[18] and by Fareed and colleagues,[19] have been unrewarding to date, both in our own laboratory and in an investigation by Karen G. Barnett, PhD, Hybridtech, Inc, San Diego (oral communication, September 1987). We have previously reported an initial impression that intralymphatic immunotherapy produces substantial augmentation of the CD4+ T-cell phenotype.[9,11] A further analysis indicates that this impression is confirmable,[20] and, notably, the more substantial increases of this subset also correlated significantly with the clinical response. Immunologic studies in progress may uncover or clarify further the underlying host-defense mechanisms and will be further evaluated in a separate report.

Our experience suggests that the method of intralymphatic immunotherapy is reasonably safe and technically feasible. It is hard to explain the regressions observed by mechanisms other than host immune responses engendered by the stimulus of the intralymphatic vaccine; understanding this process and modifying it to provide better and more durable responses remain a compelling challenge for further work.

Although one of us (C.L.W.) had previously initiated a similar program at a major academic center, we had some initial concerns about the feasibility of implementing this novel investigation at a community hospital. These concerns were not manifest in reality, and the community support of the program was gratifying. How best to further evaluate the method and to introduce it into clinical practice deserves attention. We are currently engaged in a clinical trial to evaluate a possible role for interleukin 2 in conjunction with this technique of active specific intralymphatic immunotherapy.

REFERENCES

1. Wiseman C, Rao VS: Tumor immunology and immunotherapy, chap 46, *In* McKenna RJ, Murphy GP (Eds): Fundamentals of Surgical Oncology. New York, Macmillan, 1986, pp 936-954

2. Juillard GJF, Boyer PJ, Yamashiro CH: A phase I study of active specific intralymphatic immunotherapy (ASILI). Cancer 1978; 41:2215-2225

3. Fisher B, Gellinger RC, Kelly M, et al: Variation of macrophage migration by a factor from regional lymph node cells of breast cancer patients. Cancer 1978; 42:2096-2100

4. Hoon DSB, Bowker RJ, Cochran AJ: Suppressor cell activity in melanoma-draining lymph nodes. Cancer Res 1987; 47:1529-1533

5. Juillard GJF, Boyer PJJ, Snow HD: Intralymphatic infusion of autochthonous tumor cells in canine lymphoma. Int J Radiat Oncol Biol Phys 1976; 1:497-503

6. Jeglum KA, Young KM, Barnsley K, et al: Intralymphatic autochthonous tumor cell vaccine in canine lymphoma. J Biol Response Mod 1986; 5:168-175

7. Adler A, Gillon G, Lurie H, et al: Active specific immunotherapy of renal cell carcinoma patients: A prospective randomized study of hormono-immuno- versus hormonotherapy. J Biol Response Mod 1987; 6:610-624

8. Adler A, Stein IA, Goldfarb AJ, et al: Active specific immunotherapy of stage III breast cancer: Result of an exploratory study. Cancer Immunol Immunother 1980; 10:45

9. Wiseman C, Rao VS, Bakke A, et al: Increased T-helper lymphocytes following active specific intralymphatic immunotherapy of cancer. J Biol Response Mod 1986; 5:490-497

10. Rao VS, Wiseman C, Mazumder A, et al: Effect of cholesteryl hemisuccinate (CHS) in cell-mediated immunity in melanoma patients treated with active specific intralymphatic immunotherapy. Proc Am Assoc Cancer Res 1988; 29:409

11. Rao VS, Wiseman C, Udis B, et al: Partial characterization of two subpopulations of T4 cells induced by active specific intralymphatic immunotherapy in melanoma patients. Proc Am Assoc Cancer Res 1986; 27:325

12. Berd D, Mastrangelo MJ: Effect of low dose cyclophosphamide on the immune system of cancer patients: Depletion of CD4+, 2H4+ suppressor-inducer cells. Cancer Res 1988; 48:1671-1675

13. Skornick Y, Danciger E, Rozen R, et al: Positive skin tests with autologous tumor cells of increased membrane viscosity: First report. Cancer Immunol Immunother 1981; 11:93-96

14. § 610.12. Federal Register 1973; 38:32056

15. McCune CS, Schapira DV, Henshaw EC: Specific immunotherapy of advanced renal carcinoma: Evidence for the polyclonality of metastases. Cancer 1981; 47:1984-1987

16. Weisenburger TH, Jones PC, Ahn SS, et al: Active specific intralymphatic immunotherapy in metastatic malignant melanoma: Evidence of clinical response. J Biol Response Mod 1982; 1:57-66

17. Skornick YI, Rong GH, Sindelar WF, et al: Active immunotherapy of human solid tumor with autologous cells treated with cholesteryl hemisuccinate—A phase I study. Cancer 1986; 58:650-654

18. Ahn SS, Irie RF, Weisenburger TH, et al: Humoral immune response to intralymphatic immunotherapy for disseminated melanoma: Correlation with clinical response. Surgery 1982; 92:362-367

19. Fareed GC, Mendlaz E, Sen A, et al: Novel antigenic markers of human tumor regression. J Biol Response Mod 1988; 7:11-23

20. Wiseman C, Rao S, Kennedy P, et al: Biological correlates of objective response in autologous active specific intralymphatic immunotherapy in human malignancy (AASILI). Proc Soc Biol Ther 1988; III-7:19

Exhibit 13

C-217*

AUGMENTATION OF DELAYED-TYPE HYPERSENSITIVITY (DTH) TO TUMOR-ASSOCIATED ANTIGENS BY TREATMENT WITH AUTOLOGOUS TUMOR CELL VACCINE PRECEDED BY CYCLOPHOSPHAMIDE (CY). David Berd, Henry Maguire and Michael Mastrangelo. Fox Chase Cancer Center, Philadelphia, PA 19111, Hahnemann Medical College, Philadelphia, PA 19102

CY pretreatment augments the development of DTH to a primary exogenous antigen in patients with advanced cancer. In an ongoing study, we are testing the hypothesis that CY can augment DTH to tumor associated antigens as well. We obtained tumor tissue from patients with advanced cancer who had an easily resectable metastatic deposit. Tumors were dissociated with collagenase and DNase and the cells were cryopreserved in human serum. Patients were skin-tested by i.d. injection of 10^6 live, autologous tumor cells (TC) and also with 3 x 10^6 similarly-cryopreserved blood mononuclear cells (MNC); DTH responses (mean diameter induration) were measured at 48 h. Two weeks later they were given CY 300 mg/M^2 IV. Three days after CY, patients were injected i.d. with a vaccine, consisting of TC (10-30 x 10^6 live cells) mixed with 0.1 ml Glaxo BCG. Seventeen days later, they were skin-tested again; pre- and post-treatment DTH responses were compared. So far, 6 patients have been entered on the study (5 melanoma, 1 breast Ca). Five have completed pre-treatment skin testing and the DTH responses (mm) were: TC - 5,0,4,0,6; MNC - all 0. Two patients have completed at least one immunization and testing. In patient #1, DTH to TC increased after treatment from 5 to 14 mm. Patient #2 had no DTH to TC before treatment, but had a 12 mm response after treatment. Mechanically dissociated TC elicited a response similar to that elicited by enzyme-treated TC. CY vaccine did not augment DTH to MNC. Thus, autologous TC-BCG vaccine preceded by CY can result in development or augmentation of DTH to tumor-associated antigens.

C-218*

COMBINED MODALITY THERAPY OF ADVANCED NODULAR LYMPHOMAS (NL) THE ROLE OF NONSPECIFIC IMMUNOTHERAPY (MBV) AS AN IMPORTANT DETERMINANT OF RESPONSE AND SURVIVAL. S.Kempin, C.Cirrincone, J.Myers, B. Lee III, D.Straus, B. Koziner, Z.Arlin, T.Gee, R.Mertelsmann, C.Pinsky, E.Comacho, L.Nisce, L.Old, B.Clarkson, H.Oettgen, Memorial Sloan-Kettering Cancer Center, New York, N.Y. 10021.

Since June 1976 56 patients (pts) with NL(Stages II-IV) were treated with the NHL-4 protocol, consisting of two sequential regimens: I-Thiotepa, Vincristine, Chlorambucil and Prednisone (6-12 cycles) followed by II-Cyclophosphamide, Adriamycin, Melphalan and Prednisone (4 cycles)(ASCO 22:514, 1981). Radiotherapy (RT) was administered to initial areas of bulky disease at the conclusion of chemotherapy or to nodal/extranodal sites responding only partially to chemotherapy. MBV, a heat killed preparation of S.pyogenes and Ser.marcescens, was administered in a randomized fashion, subcutaneously, one week prior to each chemotherapy cycle, to 26 pts. MBV was administered up until the completion of regimens I and II, then discontinued. The overall complete (CR) and partial response rates were 62% and 32% respectively. The CR rate of vaccinated patients was 85% vs. 44% for nonvaccinated pts. The survival duration of pts treated with MBV is significantly better than nonMBV pts (P=.029); however, with a median follow up of 34.3 months no plateau has yet been reached for either group. Relapses have occurred in 20% of MBV-CR pts vs. 42% of nonMBV CR pts. Of the 10 CR pts who have relapsed, only 5 pts have received RT and all relapses occurred in nonirradiated previously "uninvolved" sites. Including PR pts, 83% relapsed or progressed in previously involved/unirradiated sites. This trial suggests that MBV may have a significant therapeutic role in this combined modality approach. Whether longer term maintenance immunotherapy or more aggressive radiotherapy (i.e. total nodal) should be used can only be determined by long term comparative randomized trials.

C-219

ACTIVE SPECIFIC IMMUNOTHERAPY IN MALIGNANT MELANOMA. ECOG PILOT STUDY PE-680. J.E. Harris, A.C. Hollinshead, B. Fuller, and S. Nisius. Rush Medical College, Chicago and Geo. Washington U. Med. Ctr., Washington.

Sixteen patients (pts) with disseminated malignant melanoma who had failed at least one course of chemotherapy were treated by intradermal vaccinations with 0.2 ml of melanoma cell membrane extracted tumor-associated antigens (TAA) + 0.2 ml Freund's Complete Adjuvant (FCA) given monthly up to 3 times. TAA was purified from a soluble membrane pool obtained from melanoma cells metastatic to lung and liver. TAA was obtained after gel filtration and separation by preparative polyacrylamide gel electropheresis (PAGE) and were analyzed by discontinuous-gradient PAGE. Of 15 evaluable pts. (10 males, 5 females; median age 58 years (yrs), range 32-70 yrs.) 7 progressed following 1 vaccination dying 3.0, 0.5, 1.0, 2.5, 5.0, 4.0 and 0.5 months (mos.) later; 4 pts progressed following 2 vaccinations; 3 dying 4.5, 1.0 and 2.0 mos. later, with 1 currently alive at 5.0 mos.; 4 pts progressed following 3 vaccinations; 3 dying 3.0, 5.5 and 1.5 mos. later with 1 currently alive 5.5 mos. later. One partial remission of disease (complete regression of soft tissue skull metastasis with no change in inguinal node metastatic disease) was seen following the second vaccination in a pt. who then relapsed and died 7 weeks after the 3rd vaccination. There was no relationship between tolerance of, or ability to complete the 3 vaccination regimen and (i) performance status, (ii) extent and nature of metastatic disease, (iii) amount of prior cytotoxic drug therapy. Patients receiving 3 vaccinations had longer disease-free intervals between primary resection and tumor recurrence than did those receiving 1-2 vaccinations. Toxicity: transient pain at sites of vaccination and temperature elevation (99-100°F) on the days of vaccination. All vaccination sites ulcerated; none became infected; all crusted over. This Phase I study establishes that extracted tumor antigen in FCA may be given to malignant melanoma pts. with tolerable morbidity. It has antitumor activity and may be useful for immunoprophylaxis in the adjuvant setting.

C-220

EFFECT OF CHEMOTHERAPY ON MONOCLONAL ANTIBODY-DEFINED LEUKOCYTE SUBSETS IN SOLID TUMOR CANCER PATIENTS. Donald P. Braun, Jules E. Harris and Samuel G. Taylor III. Rush Medical College, Chicago, Illinois.

Percentages and numbers of T cells, monocytes and T cell subsets were measured in peripheral blood mononuclear cells (PBMC) from solid tumor cancer patients receiving chemotherapy. The patient population consisted of: 1 patient with breast cancer who received adjuvant chemotherapy with melphalan and 5-fluorouracil; 8 patients with non-small cell lung cancer who received mitomycin C, cisplatin and vindesine, 3 patients with small cell lung cancer who received cytoxan, adriamycin and VP-16, 1 patient with melanoma who received methyl-CCNU, and 1 patient with colon carcinoma who received cytoxan, oncovin and methotrexate. The following leukocyte subsets were enumerated: (i) T cells by sheep erythrocyte rosetting, (ii) T cells by OKT.3 monoclonal antibodies, (iii) helper/inducer T cells by OKT.4 monoclonal antibodies, (iv) suppressor/cytotoxic T cells by OKT.8 monoclonal antibodies, (v) monocytes by latex ingestion and (vi) OKM.1 + cells by monoclonal antibodies. Levels of phytohemagglutinin-induced (PHA) DNA synthesis were measured to determine whether drug-induced changes in leukocyte subsets were associated with changes in immune function. In all of the patients studied, absolute numbers of lymphocytes and E rosetting cells declined following drug treatment; there was no difference in the extent of depression or rate of recovery in any T cell subset. The percentage of latex-ingesting PBMC and OKM.1 + cells were found to decline in 6 patients following their drug treatment. PHA responsiveness in PBMC from 9 to 10 drug treated cancer patients improved to at least 150% of pretreatment levels of function following therapy. That was associated in 4 instances with a decline in percentages of latex ingesting cells and OKM.1 + cells but was never associated with selective changes in T cell subsets. Combination chemotherapy does not differentially affect the percentage of T cell subsets but may sometimes depress monocyte percentages in the PBMC of treated cancer patients.

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NZ 406 ENHANCED EXPRESSION OF MHC MOLECULES AND STIMULATION OF AUTOLOGOUS TUMOR INFILTRATING LYMPHOCYTES FOLLOWING TRANSDUCTION OF MELANOMA CELLS WITH IFN-γ GENES, Masahiro Ogasawara and Steven A. Rosenberg, Surgery Branch, NCI, NIH, Bethesda, MD 20892

Gene Therapy for cancer is being tested in clinical trials by using tumor infiltrating lymphocytes (TIL) or tumor cells modified by the insertion of genes coding for IL-2 or TNF-α. In the present study, we investigated the feasibility of transducing human tumor cells with genes coding for IFN-γ or IFN-α, which are two other cytokines that can enhance host antitumor immune responses.

Tumor cells from twelve melanoma and two renal cell carcinoma patients were transduced with IFN-γ retroviral vectors. In both IFN-γ secreting and non-secreting tumor lines, the cell surface expression of HLA class I and class II molecules increased following transduction. However the magnitude of the increase in MHC expression appeared to be greater in tumor lines secreting IFN-γ. Northern blot analysis showed IFN-γ transcripts only in IFN-γ transduced cells. The amount of RNA transcribed correlated roughly with IFN-γ secretion.

Two melanoma cell lines were successfully transduced with an IFN-α retroviral vector. Melanoma cells transduced with the IFN-α gene transcribed IFN-α RNA and secreted large amounts of IFN-α. In contrast to cells transduced with the IFN-γ gene, the expression of HLA class II molecules was not increased in IFN-α -transduced cells.

Finally, we tested the ability of HLA.DR+ melanoma cells, which had been transduced with the IFN-γ gene, to stimulate specific cytokine release by autologous CD 4+ TIL. Both GM-CSF and IFN-γ were secreted when the lymphocytes and tumor cells were cultured together but not when they were cultured alone or with control tumor cells. These results suggest that the HLA.DR molecules newly expressed on the transduced cells promoted antigen presentation and T cell responses against the transduced tumor cells. The insertion of IFN-γ genes into melanoma cells may be useful either for active immunization against melanoma or for the generation of TIL to be used in adoptive immunotherapy.

NZ 407 RETROVIRAL MEDIATED GENE TRANSFER OF IL-2 DECREASES TUMORIGENICITY IN MURINE B CELL LYMPHOMA. Orchard P.J.[1,2], Katsanis E.[1], Gorden K.[1], May C.[1], McIvor R.S.[2] and Blazar B.R.[1,2] [1]*Department of Pediatrics, Division of Bone Marrow Transplantation and* [2]*Institute of Human Genetics, Department of Laboratory Medicine and Pathology, University of Minnesota, 55455*

The potential to increase immune responsiveness against otherwise minimally immunogenic malignancies by the transfer of cytokine genes into tumor cells has been of great interest. We have examined the effect of endogenous production of Interleukin-2 (IL-2) on BDL-2, a murine B cell lineage lymphoma, by transducing BDL-2 with a retrovirus (LIL2SN) we have constructed containing the human IL-2 cDNA under transcriptional regulation of the Moloney long terminal repeat, the SV40 internal promoter and the neomycin phosphotransferase gene. BDL-2 clones transduced with LIL2SN were isolated by limiting dilution in G-418, and have been shown to secrete 0.2 - 88.5 U IL-2/10^6 cells/mL/24 hours (mean 29.5 U/mL) by ELISA. No changes in phenotype (MHC class I, CD2, CD5, B220, ICAM-1 or surface IgG) or variation in the rate of proliferation were observed following retroviral transduction and expression of IL-2. Intravenous (iv) or intraperitoneal (ip) injections of this IL-2 secreting BDL-2 clone in syngeneic Balb/c mice resulted in significantly increased median survival time (MST) when compared to controls ($p< 0.03$ and $p< 0.001$, respectively). Immunization with irradiated IL-2 secreting tumor cells subcutaneously (sc) resulted in enhanced survival ($p< 0.0002$) following live tumor challenge with the parental BDL-2 line 14 days later. In a minimal residual disease model, iv injection of 10^5 BDL-2 cells followed on day 12 by sc administration of 10^7 irradiated IL-2 secreting cells resulted in a significant ($p< 0.01$) improvement in MST. In vitro ^{51}Cr release assays demonstrated sensitivity of BDL-2 to both natural killer (NK) populations and activated cytotoxic T cells. In vivo depletions of $CD4^+$ cells (RL 172 antibody) $CD8^+$ cells (2.43) and NK cells (anti-asialo GM_1) were performed. Depletion of $CD8^+$ and NK cells resulted in decreased survival in mice inoculated with IL-2 secreting tumor cells iv, compared to controls and to mice depleted of $CD4^+$ cells. This implicates both cytotoxic T cells and NK cells as important in the resistance of Balb/c mice to a IL-2 secreting BDL-2 tumor line in vivo.

NZ 408 EXPRESSION OF SYNGENEIC MHC CLASS II GENES IN MELANOMA CELLS INHIBITS METASTATIC DISEASE. S. Ostrand-Rosenberg and Noelle Patterson, Dept. of Biology, University of Maryland, Baltimore, MD 21228.

Previous studies have established that transfection of syngeneic MHC class II genes into constitutively class II^- mouse sarcoma cells produces an immunogenic tumor (SaI/A^k) which is rejected by the autologous host, and which effectively immunizes the host against a challenge of wild type class II^- tumor. We have hypothesized that the SaI/A^k transfectants induce protective immunity because they function as antigen presenting cells (APC) for endogenously synthesized tumor peptides, and thereby stimulate tumor-specific T_h cells, by-passing the need for professional APC. In the present study we demonstrate that immunization with MHC class II gene transfected tumor also protects the autologous host against subsequent metastatic disease. The C3H-derived ($H\text{-}2^k$) K1735 melanoma gives high levels of spontaneous (subcutaneous inoculation) and experimental (intravenous inoculation) metastastases in syngeneic C3H mice. In order to test the protective potential of MHC class II^+ K1735 cells, wild type K1735 tumor cells were transfected with syngeneic A_a^k, A_b^k MHC class II genes, and/or neo^R gene, and 3 clones expressing high levels of I-A^k molecules selected (K1735/A^k clones). At tumor doses ranging from 10^3 to 5 X 10^5 i.v., the class II^+ transfectants give 5-10 fold fewer lung metastases than their wildtype class II^- or neo^R alone counterparts. C3H mice inoculated subcutaneously with class II^+ K1735 cells have significantly lower frequencies of primary tumors and spontaneous metastases, as compared to mice receiving wild type K1735 inocula. We have also tested the ability of K1735/A^k cells to immunize against wild type tumor. Autologous C3H mice immunized with fixed class II^+ K1735 cells and challenged i.p. 1-3 months later with wild type K1735 tumor have greatly reduced primary tumor growth and spontaneous metastasis formation relative to naive, unimmunized recipients. These studies indicate that transfection and expression of syngeneic MHC class II genes significantly reduces the metastatic potential of a mouse melanoma, and provides an immunization stategy for protecting against subsequent metastatic disease.

NZ 409 THE RETROVIRAL VECTOR MFG ALLOWS HIGH EFFICIENCY TRANSDUCTION OF HUMAN PROSTATE CANCER CELLS: IMPLICATIONS FOR GENE THERAPY OF PROSTATE CANCER. Martin G. Sanda, Sujatha Ayyagari, Liz Jaffee, Drew M. Pardoll, Richard C. Mulligan, and Jonathan W. Simons, Oncology Center and Brady Urological Institute, Johns Hopkins Hospital, Baltimore, MD, and the Whitehead Institute, MIT, Cambridge, MA.

We addressed the feasibility of gene therapy for human prostate cancer using MFG, an amphotrophic and replication defective retroviral vector lacking the gag, pol, and env genes. First, conditions optimizing transduction efficiency using MFG-lacZ (containing the gene encoding beta-galactosidase) were identified with transduction of four long term human prostatic adenocarcinoma cell lines. DEAE-dextran dose and virus-target coincubation titration demonstrated optimum transduction efficiency at 80ug/ml of DEAE-Dextran with 5 hours of target exposure to viral supernatant. Using these conditions, 21-82% transduction efficiency was achieved. We then used these conditions to transduce prostate cancer cells from patients undergoing radical prostatectomy. Cultured cells derived from surgical specimens were confirmed to be of prostatic epithelial origin by immuno-histochemical detection of luminar epithelium specific cytokeratin 18, and by detection of Prostate Specific Antigen (PSA) secretion by these cells. 200 - 300 fold expansion was achieved in vitro prior to senescence of cultured cells. Efficient transduction by MFG-lacZ of such primary culture prostate cancer cells from 7 consecutive patients (transduction efficiency range, 4.3% to 50%; median, 15.2%) demonstrates the feasibility of using MFG in genetic therapy for prostate cancer.

Exhibit 14

Volume 145 April 1991 Number

The Journal of UROLOGY®

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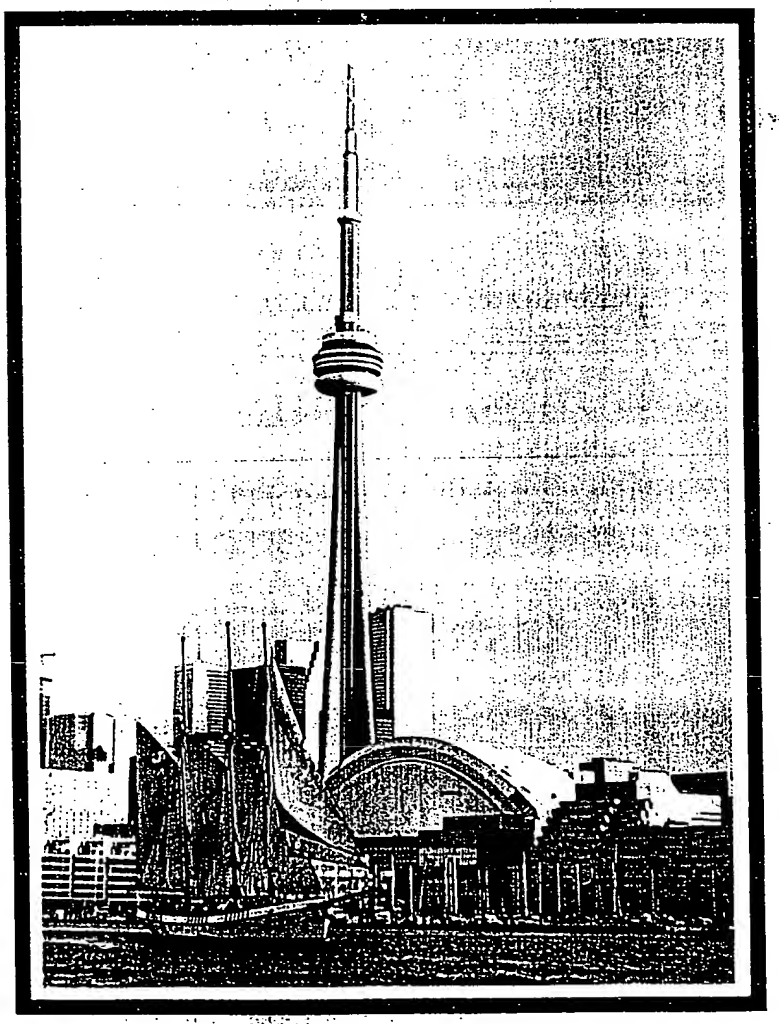

THE CN TOWER

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Supplement to The Journal of Urology

Exhibit 15

321

THE ENDOCUT NEEDLE: ENDOSCOPIC CORE BIOPSY WITHOUT NEEDLE TRACK SEEDING. *Chris J Parker, *Brian R Birch and Ronald A Miller. London, UK. (Presentation to be made by Dr Birch).

The Endocut Needle (Cook Urological) is the first spring loaded, automatic, core biopsy needle which can be passed via the instrument channel of a cystoscope, nephroscope or laparoscope. Biopsy procedures can be carried out under sedation and local anaesthesia as day-cases. A prospective study of 10 consecutive patients requiring prostatic biopsy was carried to assess the accuracy and acceptability of this new instrument.

After preliminary cystoscopy prostatic biopsies were taken x2 with the endocut needle (EN) per urethram (direct vision) and x2 with a Trucut needle (TN) per rectum (blind). Four patients subsequently underwent prostate resection (TURP) and the material so obtained was compared with that from earlier EN and TN biopsy.

Prostatic tissue was obtained in all (20/20) cases of EN biopsy but fat/fibrous tissue only was reported in 3/20 TN biopsies. EN biopsy histology was compatible with TURP material in all 4 cases. TN biopsy agreed in just 3/4 cases missing one diagnosis of carcinoma. EN biopsy was preferred to TN biopsy by all patients

It is evident that EN biopsy is accurate (an echogenic needle is available where ultrasound localisation is required), preferred by patients, reduces the risk of sepsis and operator injury and provides core samples suitable for histological examination. In the prostate this is achieved without the risk of needle-track seeding. Synchronous endoscopic and histological assessment of prostate (or bladder) tumours can then be performed as an out-patient.

322

A NOVEL MULTIDRUG RESISTANCE-1 RELATED PRODUCT SYNTHESIZED EXCLUSIVELY DURING THE REGRESSION OF THE RAT VENTRAL PROSTATE GLAND. Ilan J. Leibovitch, Carl A. Olsson, Benad Goldwasser and Ralph Buttyan, New York, NY and Tel Hashomer, Israel (Presentation to be made by Dr. Leibovitch).

The rat ventral prostate gland is an outstanding model to characterize the molecular pathway by which programmed death (apoptosis) is activated. Castration rapidly induces apoptotis in prostatic epithelial cells. As with other cell activities, apoptosis requires the synthesis of proteins and RNA; thus we can surmise that some product(s) made after castration is needed for the cell to die. We have already described constitutive gene products that are induced during regression. Other gene products have been identified (sulfated glyco-protein-2 and the Yb1 subunit of glutathione S-transferase) that are so intensely induced they seem specific for dying cells. Our studies on the role of multidrug resistance (MDR) genes in the prostate has allowed us to identify another member of this latter category, a gene product related to MDR-1 P-glycoprotein. Low stringency hybridization of Northern blots containing mRNA from regressing ventral prostates shows the intense induction (150-fold) of a 2 kb transcript (compared to 4.9 kb for MDR-1 mRNA). While undetectable in normal ventral prostate, the expression of this mRNA reaches a peak at 3-4 days after castration and declines thereafter, being barely detectable at 1 week. By screening a cDNA library made from 3-day castrate prostate mRNA, we have isolated clones for this gene and sequencing studies should determine its relationship to the normal MDR-1 product. This finding provides another member of the regression-associated products with which to identify genetic elements determining death-specific expression.

323

LYMPHOKINE-TRANSFECTED PROSTATE CANCER CELLS GENERATE AN ANTI-TUMOR EFFECT IN VIVO. *D. Branch Moody, *Charles M. Ewing and *William B. Isaacs, Baltimore, Maryland. (Presentation by D. Branch Moody)

We demonstrate an anti-tumor effect of lymphokine-transfected tumor cells against an anaplastic, rapidly growing rat prostate carcinoma. Lymphokine-secreting tumors were prepared by transfecting cDNA for Interleukin 2 (IL2) and Interleukin 4 (IL4) into the MAT LyLu (MLyLu) subline of the Dunning rat prostate carcinoma. Injection of the $7x10^5$ MLyLu cells produced lethal tumors in 100% of control rats; whereas, the same number of IL2-transfected MLyLu cells produced no tumors in 32 animals. The lymphokine-transfected cell therapy gave partial protection against subsequent injections of parental tumor. Ten of 20 animals injected with parental tumor 2-3 weeks after treatment showed no tumor growth. Animals suffered no readily apparent toxicity from the treatment. Currently, experiments are underway to further define the duration of the anti-tumor effect, maximal tumor burden which can be treated with IL2-transfected tumor, as well as potential synergy between cells transfected with IL2 and cells transfected with IL4. The results indicate that interleukin-secreting prostate cancer cells result in the rejection of an otherwise lethal prostate cancer cell inoculum and give short lived partial protection against subsequent challenge with parental tumor cells. This work is the first use of this novel lymphokine therapy of prostate cancer in animals.
(DBM supported by American Cancer Society Institutional Research Grant.)

324

INVESTIGATION OF RETINOBLASTOMA TRANSCRIPTS IN PRIMARY PROSTATIC ADENOCARCINOMA°. *J.A. Petros, *S.B. Dowton, W.J. Catalona. St. Louis, MO (Presentation by Dr. Petros)

Tissue has been preserved from 60 patients whose prostate was removed for clinically localized prostate cancer. Intraoperative selection of tumor was made, and specimens were frozen immediately in liquid nitrogen. Messenger RNA was purified by cesium chloride ultracentrafugation and by oligo dT resin affinity chromatography. Of 12 specimens investigated to date, 4 have shown absence of retinoblastoma (RB) mRNA when a cDNA probe which hybridizes with the 5' region of the RB gene (kindly provided by Dr. Takahashi, Baylor College of Medicine, Houston, TX) was used in RNA blot analysis. Integrity of mRNA and uniform RNA application to the gel were documented by re-hydridization with an actin probe. These results have not yet been reconfirmed by reanalysis of the same samples. If verified, these data suggest that the mRNA encoding the RB protein is partially or wholly absent in some primary human prostatic adenocarcinomas. Investigation is currently in progress to determine if the 3' region of the RB gene is transcribed in these tumors, or if the mRNA is completely absent. Future investigations will address the genomic mutation(s) responsible for abnormal transcription, as well as correlation with tumor behaviour and patient outcome.

°Supported by NIH Surgical Oncology Training Grant #1T32CA09621-02 and the Urological Research Foundation of Washington University

Exhibit 16

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: David BERD

Serial No.: 08/203,004 Art Unit: 1642

Filed: February 28, 1994 Examiner: Susan UNGAR

For: COMPOSITION AND METHOD OF USING TUMOR CELLS

DECLARATION OF DONALD P. BRAUN, PH.D.
UNDER 37 C.F.R. § 1.132

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

Sir:

I, Donald P. BRAUN, hereby declare and state as follows:

1. I am a citizen of the United States of America and am more than 21 years of age.

2. I presently hold the title of Administrative Director of the Medical College of Ohio Cancer Institute and Professor of Surgery at the Medical College of Ohio, 3120 Glendale Avenue, Toledo, Ohio, where I have been employed since 1999. Prior to this position, I held the positions of Director, Scientific Program Development and Professor of Medicine and Immunology/Microbiology at the Rush Cancer Institute, Rush Medical College, Chicago, Illinois. I hold a Ph.D. and M.S. degrees from the University of Illinois at the Medical Center, Chicago, and a B.S. from the University of Illinois, Urbana. I have over 25 years research experience in immunology, microbiology, and oncology, particularly cancer immunology. My qualifications are set forth more fully on the copy of my *Curriculum Vitae,* attached as Exhibit A.

3. My only connection with Avax Therapeutics, Inc. ("Avax"), is as a clinical researcher. I understand Avax has licensed certain patents and patent applications by Dr. David Berd (solely or with others) related to haptenization of tumor cells to generate an effective anti-tumor immunotherapy ("technology") from Thomas Jefferson University. I am not an employee or shareholder of Avax.

4. I know Dr. David Berd professionally. However, we have not collaborated on any research.

5. The law firm of Darby & Darby, attorneys for Applicant, has retained my services as an expert in connection with prosecution of these patent applications. In connection with these services, I attended and participated in a personal interview with the Examiner on

January 5, 2001. The law firm is compensating me for my services. Thus, I have no personal interest in Avax or the patent applications.

6. I have read and am familiar with Berd et al., Proc AACR 1989;20:382 (hereinafter "Berd 1989"; a copy is attached as Exhibit B). In particular, it is my understanding that the claims of the above-identified application have been rejected in part because the Examiner believes that Berd 1989 teaches a successful method of inducing an antitumor response comprising regression of a metastatic melanoma tumor by administering cyclophosphamide prior to autologous, irradiated, DNP-conjugated melanoma cells in combination with BCG.

7. In my view as one of skill in the art in this field, the Berd 1989 abstract does not describe successfully treating melanoma tumors with a haptenized melanoma tumor cell immunotherapy vaccine. The abstract, like most of the abstracts presented at the AACR meetings, optimistically reports preliminary observations from a new protocol. Because the abstract omits certain details, and because by its own terms the results are preliminary, one of ordinary skill in the art would not be able to conclude from this Abstract that one could effectively treat melanoma, much less any other type of cancer. Nothing in the Berd 1989 abstract suggests that this approach addresses fundamental questions of tumor vaccination (*e.g.*, as posed in a 1993 review on tumor vaccination written by myself and Jules Harris, M.D. for the Biotechnology Journal (Volume 1, No. 3), entitled "Cancer-Concept to Clinic" (Exhibit C)); which type of immune response are most important in a host response to cancer (Exhibit C, p. 28 and Table 1); whether whole cells or extracts should be used (Id., pp. 28-29); whether to use adjuvants or cytokines (Id., p. 29); and whether an

antitumor response would lead to autoimmunity (Id.). Furthermore, with respect to whole cell vaccines, whether to use autologous or syngeneic cells; fresh surgical specimens or cell lines; irradiation; reproducibility; and other factors (Id. P. 29, Table 2). The haptenization protocol of the Berd 1989 Abstract not only fails to address these variables, but also raises a new issue. Consequently, in 1989, one of skill in the art would not have viewed Berd 1989 as establishing an effective protocol for cancer immunotherapy.

8. By way of background, as described during the interview, early work on developing tumor vaccines in animal models yielded successes far beyond the reality for humans. Animals used in these models are typically immunocompetent, and the tumor cell lines (unlike spontaneous tumors) bear one or more strongly immunogenic antigens. Under these circumstances, the ability to generate an immune response cannot be viewed as particularly surprising. Unlike animal models, human cancer patients are typically immunosuppressed, whether from the tumor or chemotherapy. Spontaneous human tumors are weak immunogens. Thus the trick is to determine how to break tolerance and elicit immunity in a <u>human</u> subject. In 1992, Hanna and colleagues proposed one route, albeit based on animal data; but their results were inconclusive (Exhibit C, p. 30). Berd and colleagues offered another approach, pretreatment with cyclophosphamide to inhibit suppressor T lymphocytes (Id.) In the context of these multiple approaches, it was, in 1989, unknown and unknowable whether haptenization was a viable approach to elicit immunity to unhaptenized melanoma cells, much less that the approach could have therapeutic potential.

9. In my view as one of skill in the art in this field, the Berd 1989 abstract does not provide a definitive protocol. The description of the vaccine is ambiguous, stating that 10-25 million cells are used. It does not state if these are given as a single injection or divided into multiple sites, nor does it specify the route of administration (*e.g.*, intradermal, subcutaneous, or intramuscular). It does not specify if the injections are given in proximity to tumor sites or even directly into the tumor site (a location that one familiar with the literature at the time would assume from a reading of this abstract). It does not state how conjugation to DNP was performed or the extent of tumor cell substitution. It does not specify the ratio of tumor cells to BCG microorganisms. It also does not describe the schedule of vaccination beyond stating that vaccine or DNCB sensitization occurred 3 days following low dose cyclophosphamide i.v. administration. The statement "after 2 vaccine treatments (8 weeks)" is totally ambiguous. It is not clear if this represented a point 4 weeks following vaccine #2, 3 weeks following vaccine #2, 2 weeks following vaccine #2, or 1 week following vaccine #2. A vaccination schedule of every 55 days could apply to what is described as readily as any of the other schedules listed above. Hanna and Peters (Cancer Research 1978;38:204-9, attached hereto as Exhibit D) emphasize the critical importance of dose, schedule, route of administration, and ratio of viable tumor cells to BCG organisms in the outcome of autologous tumor vaccines. The Berd 1989 abstract, however, provides none of these details, nor could they be deduced. Without these details, one of ordinary skill would be unable to practice the technology predictably, and furthermore would have little incentive to view this approach as any more promising than a myriad of others.

10. ***There is no indication in the protocol that patients have developed an immune response to unmodified cells.*** The opening statement of Berd 1989 indicates that a previous method practiced by Berd using non-haptenized tumor cells induced DTH to melanoma cells. But in the Berd 1989 abstract, DTH testing was done only with DNP-modified tumor cells or DNP-modified autologous lymphocytes following patient sensitization with topical application of DNCB. The positive reactions described in the Berd 1989 abstract are not surprising given the experience of Fujiwara (J Immunol 1980;124:863-869; attached hereto as Exhibit E), Sherman (J Immunol 1979;123:501-502; attached hereto as Exhibit F), and others using haptens to sensitize hosts against haptenized target cells. However, the vaccine protocol of the invention involving intradermal injection of hapten-modified autologous tumor cells, results in DTH to autologous non-haptenized tumor cells, an event that could not have been anticipated nor expected as a result of what is described in the Berd 1989 abstract or from what was known in the literature.

11. ***There is no convincing indication that the patients described in the Berd 1989 abstract received any clinical benefit.*** The descriptions of inflammatory reactions, CD4 and CD8 infiltration, and fluid accumulation over tumor lesions is no indication of clinically significant tumor regression (defined by those practiced in the art as a greater than 50% reduction in tumor size without concomitant progression in other sites). In fact, the description of lesion changes in the patients would be expected at the time of its publication, since one would presume based on Fujiwara (Exhibit D), that the patients had been sensitized to DNCB and then injected intratumorally with DNP-modified tumor cells. A skilled immuno-oncologist would have presumed that the tumor cell vaccine, which produced the described physical changes in proximity to tumor sites after only

two vaccine treatments, had been administered by intratumoral injection as taught by others (while not practiced by the Berd protocol developed subsequent to the 1989 Abstract). The same outcome would have been seen if the patients had been sensitized to BCG and then injected intratumorally with BCG. Thus, the description of the lesion changes in the 1989 Abstract would be impossible to interpret as indicative of a clinical response to a systemic vaccine. A reader would assume that a clinically meaningful tumor regression, if present, would have been reported in the abstract, and that the absence of such a report represented uncertainty.

12. For all of these reasons, the Berd 1989 abstract does not disclose a method for the successful vaccination of cancer patients using haptenized autologous tumor cells.

13. A final basis for the above statement can be deduced from Exhibit C. As discussed above, this review cites the work by Hanna and Hoover (references 7-9), and the work by Berd et al. employing non-haptenized melanoma cells (reference 10), among a number of hopeful, even promising, research approaches to cancer immunotherapy. Had the Berd 1989 abstract been indicative of a clinically meaningful vaccine methodology, that approach would have been considered in the review as well.

14. I declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true. I further declare that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States

code and that such willful false statements may jeopardize the validity of the instant application or of any patent issued thereupon.

Respectfully submitted,

Date: 5/24/01

Donald P. Braun, Ph.D.

Enclosure:

Exhibit A:	Curriculum Vitae of Donald P. Braun, Ph.D.
Exhibit B:	Berd et al., Proc AACR1989;20:382
Exhibit C:	Braun and Harris, Biotechnol J 1993;1, No. 3.
Exhibit D:	Hanna et al., Cancer Research 1978;38:204-209
Exhibit E:	Fujiwara et al., J Immunol 1980;124:863-869
Exhibit F:	Sherman, J Immunol 1979;123:501-502

CURRICULUM VITAE

Donald P. Braun, Ph.D.

January, 2000

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SS # 355-44-2224
Married: Judy Braun
Children: Jennifer, Matthew, Bethany

EDUCATION AND TRAINING:

1972	University of Illinois, Urbana, IL, B.S. degree.
1974	University of Illinois at the Medical Center, Chicago, IL, M.S. degree.
1976	University of Illinois at the Medical Center, Chicago, IL, Ph.D. degree.

CHRONOLOGY OF EMPLOYMENT:

1976-1977	Research Associate, Department of Microbiology, University of Illinois at the Medical Center, Chicago, IL.
1977-1978	Instructor, Department of Microbiology, University of Illinois at the Medical Center, Chicago, IL
1978-1979	Research Associate, Section of Medical Oncology, Rush-Presbyterian-St. Luke's Medical Center, Chicago, IL.
1979-1980	Instructor, Department of Medicine; Assistant Professor, Department of Immunology/Microbiology, Rush Medical College, Chicago, IL.
1981-1983	Assistant Professor, Department of Medicine; Assistant Professor, Department of Immunology/ Microbiology, Rush Medical College, Chicago, IL.
1983-1987	Associate Professor, Department of Medicine; Assistant Professor, Department of Immunology/Microbiology, Rush Medical College, Chicago, IL.
1987	Associate Professor, Department of Medicine; Associate Professor, Department of Immunology/Microbiology, Rush Medical College, Chicago, IL.

1989	Associate Director, Section of Medical Oncology (for Research); Associate Professor, Department of Medicine; Associate Professor, Department of Immunology/Microbiology, Rush Medical College, Chicago, IL.
1993-1999	Director, Scientific Program Development. Rush Cancer Institute.
1993-1999	Professor of Medicine and Immunology/Microbiology.
1999-present	Administrative Director of the Cancer Institute, Medical College of Ohio
1999-present	Professor, Department of Surgery, Medical College of Ohio.

FEDERAL GOVERNMENT/PUBLIC ADVISORY COMMITTEES:

1982-1984	Member, Experimental Therapeutics Study Section, National Cancer Institute.
1983,1985	Member, Small Business Innovation Grant Review Study Section, NCI.
1985-1988	Member, Experimental Therapeutics I Study Section, NCI.
1985	Chairman, Experimental Therapeutics Special Study Section, NCI.
1985-1992	Biological Response Modifier Committee, Illinois Cancer Council
1986-present	Reviewing Member, Arizona Disease Control Research Commission.
1988-1989	Member, Chicago Leukemia Research Society.
1988-1989	Member, Small Business Innovation Grant Review Study Section, NCI.
1990, 1993	Ad Hoc reviewer, Experimental Therapeutics Study Section 1, NCI.
1991, 1992	Ad Hoc reviewer, Immunology and Immunotherapy Study Section, American Cancer Society-National Division.
1994-1998	Member, Immunology and Immunotherapy Study Section, American Cancer Society-National Division.
1999-present	Advisory Member, "Molecular Targets for therapy of Lung Cancer", National Cancer Institute/CTEP.
1999-present	Advisory Member, Ohio Cancer Incidence Surveillance System
2000	American Cancer Society Immunology and Immunotherapy Study Section, National Division-ad hoc review.

CONSULTANT POSITIONS:

Burrough's Wellcome, 1983-1984
Pfizer Pharmaceutical, 1986-1988
Boehringer Mannheim, 1991-1993
Abbott Laboratories, 1993
Institute for the Study and Treatment of Endometriosis, 1990-present
Adeza Biomedical, 1993
Imutec Corporation, 1993-present
Imutec Corporation; Chairman, Medical Advisory Board, July, 1995-present.
RxKinetic Inc.; Chairman, Medical Scientific Advisory Board. 1997-present.

COMPETITIVE EXTRAMURAL GRANT AWARDS: (note: as Principal Investigator or co-Principal Investigator only)

1. "Cancer Drug Effects on Patient Suppressor Cells". Source: NIH/NCI # CA27598 Period of Support: 09/01/80-03/31/87; as co-Principal Investigator.

2. "Immune Testing in Lung CA During Specific Immunotherapy". Source: NIH/NCI # CA26138. Period of Support: 07/01/80-06/30/83; as co-Principal Investigator.

3. "A Phase I Clinical Trial of Natural and Recombinant Interleukin-2 (IL-2). Source: NIH/NCI # RFA No-1-CM47667-BRM-MA01. Period of Support: 09/30/84-03/31/87. Subcontract from the Illinois Cancer Council; as Laboratory Principal Investigator for Rush Component.

4. "Phase IB and/or Phase II Clinical Trial of Natural and Recombinant Interleukin-2 (IL-2). Source: NIH/NCI # RFA No-1 CM47667-03 BRM-MA-04. Period of Support: 09/29/85-02/28/89. Laboratory Principal Investigator for Rush Component.

5. "Arachidonate Metabolism in Cancer Patient Macrophages". Source: NIH/NCI # CA41741.Period of Support: 07/01/88-06/30/92; as Principal Investigator.

6. "LAK Function in Tumor-Infiltrating Leukocytes of Cancer Patients". Source: American Society of Clinical Oncology- 1990 Young Investigator Award to E. Staren, M.D.; as Mentor.

7. "Cancer Patient Macrophage Function in Tumor Environments". Source: NIH/NCI #CA58922. Period of Support: 12/31/92-07/01/96 as Principal Investigator.

8. "American Cancer Society New Investigator Grants in Cancer Research". Source: American Cancer Society. Period of Support: 06/31/95-07/01/97 as Principal Investigator.

9. "Cancer Drug Modulation of Tumor Sensitivity to Macrophages". Source: NIH/NCI, period of support: 12/01/00-11/30/05, as Principal Investigator. status-pending.

10. "Cyclooxygenase Metabolism in Cancer Patient Psychoneuroimmunology". Source: American Cancer Society. Period of Support: 01/01/01-12/31/06, as Principal Investigator. Status-pending.

NONCOMPETITIVE EXTRAMURAL FUNDING: (as principal or co-principal investigator)

1. "The Effect of CGS13080, CGS14854 and CGS53913 on Arachidonic Acid Metabolites and Immune Status of Patients with Solid Tumors". Source: Ciba-Geigy. Period of Support: 10/01/87-09/30/88; as Principal Investigator.

2. "Phase III Protocol for Evaluation of Combined Modalities in the Treatment of Colonic Carcinoma with Positive Nodes, Duke's C, Surgical Resection Alone vs. Postoperative Immunotherapy followed by Chemotherapy". Source: Litton Institutes. Period of Support:06/30/88-present; as Laboratory Principal Investigator for Rush.

3. "Immunologic Testing and Limited Feldene Administration to Patients with Upper Aerodigestive Tract Squamous Cancer". Source: Pfiser Laboratories, Pfiser Inc. Period of Support: 10/01/89-06/30/91; as Principal Investigator.

4. "Macrophage Function in Women with Endometriosis". Source: Sterling International. Period of Support: 04/01/90-10/01/91; as Principal Investigator for Rush component.

5. "Macrophage Regulation of Endometrial Cell Growth in Women with Endometriosis". Source: Sterling International. Period of Support: 03/01/92-10/01/93; as Principal Investigator for Rush component.

6. "Mechanisms for Modulation of Macrophage Tumoricidal Function in Cancer Patients by Virulizin". Source: Imutec Corporation. Period of Support: 02/01/94-12/01/95; as Principal Investigator.

7. "Immunological Modulation in Pancreatic Cancer Patients treated with Virulizin". Source: Imutec International. Period of Support: 06/30/96-12/31/98; as Principal Investigator.

8. "Modulation of Macrophage Cytolytic Function by Virulizin in Endometriosis". Source: Imutec International. Period of Support: 06/30/96-12/31/97; as Principal Investigator.

9. "Interaction of HIP/PCA particles with leukocytes from Cancer Patients". Source: RxKinetix. Period of Support: 01/01/98 – 08/31/2001; as Principal Investigator.

10. "Endothelin Regulation of Tumor Proliferation and Apoptosis in Human Intracranial Malignancy". Source: Abott Laboratories. Period of Support (pending-to begin in 2000); as Principal Investigator.

11. "Amelioration of TNFα effects in endometriosis by Enbrel". Source: Immunex Corp. Period of support: 01/01/00-06/30/01. As Laboratory Principal Investigator.

11. "H11 binding to human cancer cells". Source: Novopharm Biotechnology. Period of Support: 05/01/00-09/01/01. As Principal Investigator.

HONORS:

USPHS Immunology Trainee, 1973-1974.
USPHA Oncology Trainee, 1974-1976.
Milan V. Novak Award, University of Illinois, Department of Microbiology, 1977.
Who's Who in Cancer Research, 1985
American Men and Women in Science, 1988
1st place award for original research, American Fertility Society, 1992.
Chairman, Poster-Discussion Session, AACR, 1994.
Chairman, Immunology Plenary Session, Vth International Conf. on Endometriosis, 1996.
Chairman, Rationale for Immunotherapy in Endometriosis: VI World Congress on Endometriosis, Quebec City, Canada, 1998.

ACTIVE MEMBERSHIPS:

American Association for Cancer Research
American Chemical Society
American Association for the Advancement of Science
New York Academy of Science
Society of Biology Response Modifiers
American Fertility Society
American Society of Reproductive Medicine

PUBLICATIONS:

BOOKS EDITED:

1. Prostaglandin Inhibitors in Cancer Immunology and Immunotherapy. eds. JE Harris, DP Braun and KM Anderson. CRC Press, Boca Raton, FLA, 1994.

REVIEWS AND BOOK CHAPTERS:

1. Dray S, and Braun DP: Some perspectives on the transfer of cell mediated immunity by immune RNA. Mol Cell Biochem 25:15, 1979.

2. Braun DP, and Harris JE: Serial immune function testing to predict clinical disease relapse in patients with solid tumors. Cancer Immunol Immunother 15:165, 1983.

3. Harris JE, and Braun DP: The effect of cytotoxic drugs on immunoregulatory cell function in solid tumor cancer patients. Clin Immunol Newsletter 5:113-116, 1984.

4. Braun DP, and Harris JE: Effects of cytotoxic chemotherapy on immune function in cancer patients. In: Proceedings of the 3rd International Symposium of the Evaluation of the Immunomodifiers, 1984.

5. Braun DP, and Harris JE: Modulation of the immune response by chemotherapy. In: The Modulation of Immunity. Mitchell MS (ed), Oxford: Pergamon Press, 1985.

6. Braun DP, and Harris JE: Effects of cytotoxic chemotherapy on immune function in cancer patients. Cancer Treat Symp 1:19-26, 1985.

7. Braun DP, and Harris JE: Cancer chemotherapy and its impact on the immune system. In: Fundamentals of Cancer Chemotherapy. Carter SK, and Hellman K (eds), New York: McGraw-Hill, pp 77-97, 1986.

8. Von Roenn J, Harris JE, and Braun DP: Suppressor cell function in solid tumor cancer patients. J Clin Oncol 5:150-159, 1987.

9. Dmowski WP, Braun DP and Gebel H: Endometriosis: Genetic and Immunologic Aspects. in: Current Concepts in Endometriosis. 2nd International Symposium on Endometriosis. Alan R. Liss, Inc. New York, p 99-122, 1989.

10. Dmowski WP, Braun DP and Gebel H: The Immune System in Endometriosis. in Modern Approaches to Endometriosis. J. Rock ed. Kluwer Academic Publishers, p 97-111, 1991.

11. Braun DP and Groenwald SL: The Immune System and Cancer. in Cancer Nursing: Principles and Practice. third edition. Groenwald SL and Goodman M eds. Jones and Bartlett, Boston, MA, pp 70-85, 1993.

12. Harris, J.E. and Braun, D.P.: Tumor Vaccination. in Cancer: Concept to Clinic. Medical Publishing Enterprises. Fair Lawn, NJ; E. Borden, ed. pp. 28-31, 1993.

13. Braun DP: The Impact of Prostaglandins on Cancer Patient Immunity. in Prostaglandin Inhibitors in Tumor Immunology and Immunotherapy. Harris JE, Braun DP and Anderson KM, eds. CRC Press, Boca Raton, Florida, pp. 109-129, 1994.

14. Dmowski WP, Gebel HM and Braun DP. The Role of Cell-Mediated Immunity in Pathogenesis of Endometriosis. Acta Obstet. Gynecol. Scand. Suppl. 73:7-14, 1994.

15. Dmowski WP and Braun DP. Immunological Aspects of Endometriosis. Contemp. Rev. Obstet. Gynaecol. 7: 167-171, 1995.

16. Dmowski WP, Braun DP, and Rotman, C. Aspectos Immunologicos de la endometriosis. in Reproducion Humana. Remonhi, J, Simon C, Pellicer, A and Bonilla-Musoles, eds. McGraw Hill InterAmericana, Madrid, pp 195-204, 1996.

17. Braun DP and Dmowski WP. Endometriosis: Abnormal Endometrium and Dysfunctional Immune Response. Current Opinion in Obsterics and Gynecology. 10:365-369, 1998.

18. Dmowski WP, Gebel H, and Braun DP. Decreased Apoptosis and sensitivity to macrophage-mediated cytolosis of endometrial cells in endometriosis. Human Reproduction. In Press, 1999.

ARTICLES:

1. Braun DP, and Dray S: Immune RNA mediated transfer of tumor antigen responsiveness to unresponsive peritoneal exudate cells from tumor bearing animals. Cancer Res 37:4138-4144, 1977.

2. Mortensen RF, Braun DP, and Gewurz H: Effects of C-reactive protein on lymphocyte function. III. Inhibition of antigen-induced lymphocyte stimulation and lymphokine production. Cell Immunol 28:59-68, 1977.

3. Braun DP, Hengst J, Moykr M, and Dray S: Antitumor immunity in strain 2 guinea pigs immunized with KCl extracts of L_2C tumor cells. J Natl Cancer Inst 60:899-903, 1978.

4. Mokyr M, Braun DP, Usher D, Reiter H, and Dray S: The development of *in vitro* and *in vivo* antitumor cytotoxicity in noncytotoxic, MOPC-315, tumor cells. Cancer Immunol Immunother 4:143-150, 1978.

5. Braun DP, Mokyr M, and Dray S: Generation of anti-MOPC-315 cytotoxicity in uneducated or *in vitro* educated spleen cells from normal or MOPC-315 tumor bearing mice pretreated *in vivo* with BCG. Cancer Res 38:1626-1631, 1978.

6. Mokyr M, Braun DP, and Dray S: Augmentation of antitumor cytotoxicity in spleen cells of MOPC-315 tumor bearers. In: Cancer Immunology: Experimental and Clinical. Crispin RG (ed), p 211, 1978.

7. Mokyr M, Braun DP, and Dray S: Augmentation of antitumor cytotoxicity in MOPC-315 tumor bearer spleen cells by depletion of glass adherent cells prior to *in vitro* education. Cancer Res 39:785, 1979.

8. Mokyr M, Bennett JA, Braun DP, Hengst JCD, Mitchell MS, and Dray S: Opposite effects of different strains or batches of the same strain of BCG on the *in vitro*

generation of syngeneic and allogeneic antitumor cytotoxicity. J Natl Cancer Inst 64:339, 1980.

9. Cobleigh MA, Braun DP, and Harris JE: Age dependent changes in human peripheral blood B cell and T cell subsets: Correlation with mitogen responsiveness. Clin Immunol Immunopathol 15:162, 1980.

10. Braun DP, Cobleigh MA, and Harris JE: Selective effect of cytotoxic chemotherapy on immunoregulatory suppressor cells in solid tumor cancer patients. In: Tumor Progression. Crispen RG (ed), Philadelphia: Franklin Institute Press, pp 91-102, 1980.

11. Braun DP, Cobleigh MA, and Harris JE: Multiple concurrent immunoregulatory defects in cancer patients whose peripheral blood leukocytes exhibit depressed PHA induced lympho blastogenesis. Clin Immunol Immunopathol 17:89, 1980.

12. Cobleigh MA, Braun DP, and Harris JE: Quantitation of lymphocytes and T cell subsets (T_G and T_M cells) in disseminated solid tumor cancer patients. J Natl Cancer Inst 64:1041, 1980.

13. Paque RE, Braun DP, and Dray S: Characterization of lymphoid cell RNA which modulates specific cellular immunity. In: Second International Symposium on RNA in Development. Academia Sinics, 1980.

14. Braun DP, and Harris JE: Modulation of immune response by chemotherapy. Pharmacol Ther 14:89-122, 1981.

15. Braun DP, and Harris JE: Effects of combination chemotherapy on immunoregulatory cells in the peripheral blood of solid tumor cancer patients: Correlation with rebound-overshoot immune function recovery. Clin Immunol Immunopathol 20:143, 1981.

16. Braun DP, and Harris JE: Relationship of leukocyte numbers, immunoregulatory cell function and phytohemagglutinin responsiveness in cancer patients. J Natl Cancer Inst 67:809, 1981.

17. Braun DP, Harris JE, Maximovich S, Marder R, and Lint TF: Chemiluminescence in peripheral blood mononuclear cells of solid tumor cancer patients. Cancer Immunol Immunother 12:31, 1981.

18. Braun DP, Penn RD, Flannery AM, and Harris JE: Immunoregulatory cell function in peripheral blood of glioblastoma multiforme patients. Neurosurg 12:31, 1981.

19. DeBoer KP, Braun DP, and Harris JE: Natural cytotoxicity and antibody dependent cytotoxicity in solid tumor cancer patients: Regulation by adherent cells. Clin Immunol Immunopathol 23:133, 1982.

20. Harris JE, DeBoer KP, Vahey AL, and Braun DP: The measurement of leukocyte subsets in the peripheral blood of solid tumor cancer patients using monoclonal antibody reagents. Med Pediatr Oncol 10:185, 1982.

21. Taylor SG IV, Saffold P, Braun DP, and Harris JE: T_G cell involvement in the leukocyte adherence inhibition phenomenon. J Natl Cancer Inst 68:549, 1982.

22. Braun DP, DeBoer KP, and Harris JE: Chemiluminescence, suppression and cytotoxicity in peripheral blood mononuclear cells from solid tumor cancer patients. Cancer Immunol Immunother 14:86, 1982.

23. Harris JE, Harris ZL, and Braun DP: Effect of interferon-α on indomethacin sensitive immunoregulatory cells. In: Proceedings of the 15th International Leukocyte Culture Conference. Parker JW, and O'Brien R (eds), Chichester, England: John Wiley and Sons, Ltd., p 635, 1983.

24. Chiu K, Harris JE, Kroin J, Slayton R, and Braun DP: The immunological response of Wistar rats to intracranially implanted C-6 glioma cell line. J Neurooncol 1:365-372, 1983.

25. Penn RD, Kroin JS, Harris JE, Chiu K, and Braun DP: Chronic intratumoral chemotherapy of a rat brain tumor with cisplatin and flourouracil. Appl Neurophysiol 46:240-244, 1983.

26. Braun DP, Nisius S, Hollinshead AC, and Harris JE: Serial immune testing in surgically resected lung cancer patients. Cancer Immunol Immunother 15:114-121, 1983.

27. Braun DP, Harris ZL, Harris JE, Sandler S, Khandekar J, Locker G, Haid M, Gordon L, Shaw J, Cobleigh MA, and Gallagher P: The effect of interferon therapy on indomethacin sensitive immunoregulation in the peripheral blood mononuclear cells of renal cell carcinoma patients. J Biol Response Mod 2(3):251-262, 1983.

28. Van Epps SF, Stewart LD, Pandey JP, Fudenberg HH, Harris JE, and Braun DP: Immuno-globulin G heavy chain (Gm) allotypes in lung cancer. N Engl J Med 1983.

29. Braun DP, Harris JE, and Rubenstein M: Relationship of arachidonic acid metabolism to indomethacin sensitive immunoregulatory function and lymphocyte PGE sensitivity

in peripheral blood mononuclear cells of disseminated solid tumor cancer patients. J Immunopharmacol 6(3):227-236, 1984.

30. Braun DP, Penn RD, and Harris JE: Regulation of natural killer function by glass adherent cells in patients with primary intracranial malignancies. Neurosurg 15(1):29-33, 1984.

31. Braun DP, and Harris JE: Effects of combination chemotherapy on PGE mediated immunoregulation in the peripheral blood mononuclear cells of solid tumor cancer patients. J Biol Response Mod 3:391-396, 1984.

32. Braun DP, and Harris JE: Abnormal indomethacin sensitive suppression in peripheral blood mononuclear cells of cancer patients restricts augmentation by interleukin 2. J Biol Response Mod 3:533-540, 1984.

33. Chiu KM, McPherson LH, Harris JE, and Braun DP: The separation of cytotoxic human peripheral blood monocytes into high and low phagocytic subsets by centrifugal elutriation. J Leukocyte Biol 36:729-737, 1984.

34. Braun DP, and Harris JE: Effect of cytotoxic antineoplastic chemotherapy on immuno-regulatory leukocytes measured with monoclonal antibodies. Clin Immuno Immunopathol 33(1);54-56, 1984.

35. Leslie WT, Templeton A, and Braun DP: Kaposi's sarcoma in the acquired immune deficiency syndrome. Med Pediatr Oncol 12:336-342, 1984.

36. Braun DP, and Harris JE: Effect of chemotherapy on NK function in the peripheral blood of cancer patients. Cancer Immunol Immunother 21:240-245, 1986.

37. Braun DP, and Harris JE: Abnormal monocyte function in patients with Kaposi's sarcoma. Cancer 57:1501-1506, 1986.

38. Janus TJ, Braun DP, and Harris JE: Modulation of lymphocyte responsiveness to phyto-hemagglutinin by micromolecular fibrinogen degradation products. Clin Immunol Immuno-pathol 41:26-34, 1986.

39. Potempa LA, Gewurz H, Harris JE, and Braun DP: Stimulatory effects of the C-reactive protein subunit on monocyte function, including release of IL 1. Protides Biol Fluids 34:287-290, 1986.

40. Braun DP, Bonomi PD, Taylor SG IV, and Harris JE: Modification of the effects of cytotoxic chemotherapy on the immune responses of cancer patients with a nonsteroidal anti-inflammatory drug, piroxicam. J Biol Response Mod 6:331-345, 1987.

41. Harris JE, and Braun DP: Modulation of immune function in cancer patients. Clin Physiol Biochem 5:222-226, 1987.

42. Wiederhold MD, Ondrey F, Anderson KM, Braun DP, and Harris JE: Synthesis of eicosanoids by γ-interferon differentiated U937 cells. Clin Physiol Biochem 6:21-28, 1988.

43. McMannis JD, Fisher RI, Creekmore SP, Braun DP, Harris JE, and Ellis TM: *In vivo* effects of recombinant IL 1. I. Isolation of circulating Leu 19^+ lymphokine activated killer effector cells from cancer patients receiving recombinant IL 2. J Immunol 140:1335-1340, 1988.

44. Harris JE, and Braun DP: Modulation of *in vitro* antitumor immunity in response to the biological activators of IL 1, γ-interferon, and GM-CSF in the peripheral blood of cancer patients receiving cytotoxic chemotherapy. Ann NY Acad Med 65:16-25, 1988.

45. Braun DP, Kessler H, Falk L, Paul D, Harris JE, Blaauw B, and Landay AL: Monocyte functional studies in asymptomatic HIV-infected individuals. J Clin Immunol 8:486-494, 1988.

46. Staren ED, Braun DP, Harris JE, Bines SD, and Economou SG: Lymphokine activated killer cell induction in the peripheral blood mononuclear cells of colon cancer patients. Surg Forum 39:447-449, 1988.

47. Ellis TM, Creekmore S, McMannis J, Braun DP, Harris JE, and Fisher RI: Appearance and phenotypic characterization of circulating Leu 19^+ cells in cancer patients receiving recombinant interleukin 2. Cancer Res 48:6597-6602, 1988.

48. Braun DP, Taylor ST IV, and Harris JE: Modulation of immunity in cancer patients by prostaglandin antagonists. Prog Clin Biol Res 288:444-448, 1989.

49. Creekmore SP, Harris JE, Ellis TM, Braun DP, Cohen II, Bhoopalam N, Jassak PF, Cahill MA, Canzoneri CL, and Fisher RI: A phase I clinical trial of recombinant interleukin 2 by periodic 24 hour infusions. J Clin Oncol 7:276-284, 1989.

50. Staren ED, Economou SG, Harris JE, and Braun DP: Lymphokine activated killer cell induction in tumor infiltrating leukocytes from colon cancer patients. Cancer 64:2238-2242, 1989.

51. Braun DP, Siziopikou KP, Casey LC, and Harris JE: The *in vitro* development of cytotoxicity in response to granulocyte/macrophage colony stimulating factor or γ-interferon in the peripheral blood monocytes of patients with solid tumors. Modulation

by arachidonic acid metabolic inhibitors. Cancer Immunol Immunother. 32:55-61, 1990.

52. Harris JE, and Braun DP: The effect of aspirin and other cyclooxygenase inhibitors on antitumor immunity. In: Aspirin towards 2000 (G. Fryers, editor). pp. 45-52. Royal Society of Medicine. 1990.

53. Siziopikou KP, Harris JE, Casey L, Nawans Y and Braun DP. Impaired Tumoricidal Function of Alveolar Macrophages from Patients with Non-small Cell Lung Cancer. Cancer, 68:1035-1044, 1991.

54. Braun, D.P., Gebel, H., Rana, N, Rotman, C, and Dmowski, W.P. Peritoneal Macrophage Function in Women with Endometriosis., Fertility and Sterility 57: 1203-1210, 1992.

55. Harris, J.E. and Braun, D.P.: Abnormal immunoregulation in human cancer at the actual tumor site. In "Combination Therapies. Biological Response Modifiers in the Treatment of Cancer and Infectious Diseases. A. Goldstein, ed. Plenum Publishing, New York, pp. 197-206, 1992.

56. Harris, J.E. and Braun, D.P.: Abnormal immunoregulation and the tumor dormant state in human cancer. In "Cellular Immune Mechanisms and Tumor Dormancy" (T. Stewart, editor). CRC Press, Boca Raton, FLA, pp. 261-280, 1992.

57. Chu, E., Casey, L.C., Harris, J.E. and Braun, D.P. Suppression of the Development ofTumoricidal Function in Gamma Interferon-treated Human Peripheral Blood Monocytes by Lipopolysaccharide: The role of Cyclooxygenase Metabolites. J. of Clin. Immunol. 13: 49-57, 1993.

58. Braun, DP, Ahn, M, Chu, E, Casey, L.C., Harris, JE, Wilbanks, G, and Siziopikou, KP. The sensitivity of macrophages from Different Anatomical Sites of Cancer Patients to Arachidonic Acid Metabolism. Cancer Research 53: 3362-3368, 1993.

59. Braun, DP, Muriana M, Gebel H, Rotman C, Rana N and Dmowski WP. Monocyte-mediated enhancement of endometrial cell proliferation in women with endometriosis. Fertility and Sterility 61: 78-85, 1994.

60. Braun, DP, Gebel H and Dmowski WP. Effect of danazol in vitro and in vivo on monocyte-mediated enhancement of endometrial cell proliferation in women with endometriosis. Fertility and Sterility 62: 89-95, 1994.

61. Gebel HM, Braun DP, Rotman C, Rana N and Dmowski WP. Decreased in vitro production of IgG in women with severe endometriosis. Amer. J. Reprod. Immunol. 29: 124-130, 1993.

62. Dmowski WP, Gebel HM and Braun DP. The role of cell-mediated immunity in pathogenesis of endometriosis. Acta Obstet Gynecol Scand 159: 7-14, 1994.

63. Gebel HM, Rana N, Braun DP, and Dmowski WP. Differential Expression of VLAB1 (CD29) on monocytes from patients with endometriosis. Amer J Reprod Immunol 34: 317-322, 1995.

64. Rana N, Braun DP, Rotman C, Gebel HM and Dmowski WP. Cytokine Synthesis by peritoneal macrophages in patients with endometriosis. Fertility Sterility,65:925-931,1996.

65. Braun DP, House R, Gebel HG, Rana N, and Dmowski WP. Cytokine synthesis by peripheral blood monocytes of patients with endometriosis. Fertility and Sterility 65: 1125-1129, 1996.

66. Braun DP and Preisler HD. Cytolytic Activity of Peripheral Blood Blast Cells from Patients with Acute Myeloid Leukemia. Leukemia and Lymphoma 27: 459-467, 1997.

67. Siziopikou KP, Ahn M, Casey LC, Silver M, and Braun DP. Augmentation of impaired tumoricdal function in alveolar macrophages from lung cancer patients by coculture with allogeneic lymphocytes. Cancer Immunol. Immunother. 45:29-36, 1997.

68. Ahn M, Siziopikou KP, Casey LC, Harris JE and Braun DP. Suppression of tumoricidal function in alveolar macrophages from lung cancer patients by Interleukin-6. Cancer Immunol. Immunother. 45: 37-44, 1997.

69. Ono N, Glick R, Kroin J. Penn RD and Braun DP. Effect of IGF inhibitor on proliferation of rat C6 glioma and human glioma explants. Submitted to J. Neurosurgery.

70. Braun DP, Gebel HG, Rana N, and Dmowski WP. Cytolysis of eutopic and ectopic endometrial cells by peripheral blood monocytes and peritoneal macrophages in women with endometriosis. Fertility and Sterility 69: 1103-, 1998.

71. Gebel HG, Braun DP, Frame D, Tambur A, Rana N and Dmowski WP. Spontaneous Apoptosis in eutopic and ectopic endometrium from women with endometriosis. Fertility and Sterility 69: 1042-1047, 1998.

72. Wong PY, Staren ED, Tereshkova N, and Braun DP: Functional Analysis of Tumor-infiltrating leukocytes in breast cancer patients. J. Surg. Res. 76: 95-103, 1998

73. Wilbanks GD, Ahn M, Beck DA and Braun DP. Antitumor cytotoxicity of peripheral blood monocytes and peritoneal macrophages from patients with gynecological malignancies. Int. J. Gynecological Cancer 9: 427-432, 1999.

74. Ferdinandi E, Braun DP, Liu C, Zee B, and Ely G. Virulizin: A Review of its Antineoplastic Activity. Exp Opin. Invest. Drugs 8: 1721-1735, 1999.

75. Ortegel JW, Staren ED, Faber LP, Warren WH and Braun, DP. Cytokine Biosynthesis by Tumor-Infiltrating T Lymphocytes from Human Non-Small Cell Lung Carcinoma. Cancer Immunology and Immunotherapy, 48:627, 2000.

76. Ortegel JW, Staren ED, Faber LP, Warren WH and Braun, DP. Modulation of Autologous Tumor cytotoxicity by Tumor-infiltrating Lymphocytes from non-Small Cell Lung Carcinoma by Type 2 Cytokines and Transforming Growth Factor-β. Submitted to Journal of Cytokine Research, 2000.

77. Dmowski, WP, Ding, J, Shen, J, Rana, N, Fernandez, BB, Braun, DP. Apoptosis in Endometrial Glandular and Stromal Cells in Women With and Without Endometriosis. Submitted to Human Reproduction, July 2000.

ABSTRACTS AND LETTERS:

1. Braun DP, and Dray S: Tumor immune RNA conversion of lymphoid cells of tumor-bearing mice from being unresponsive to responsive upon challenge with tumor specific antigen. Cancer Res 17:7, 1976.

2. Braun DP, and Dray S: The effect of BCG on the *in vitro* education of normal spleen cells and of tumor bearing spleen cells. Cancer Res 19:154, 1978.

3. Hengst JCD, and Braun DP: Opposite effects of different batches of BCG on the *in vitro* generation of antitumor cytotoxicity. Cancer Res 20:67, 1979.

4. Braun DP, Cobleigh MA, and Harris JE: Explanation for rebound-overshoot recovery of immune function in cancer patients receiving cytotoxic drugs. Clin Res 27:643A, 1979.

5. Cobleigh MA, Braun DP, and Harris JE: Lymphocyte subset variation in normal aging subjects and in cancer patients. Clin Res 27:643A, 1979.

6. Cobleigh MA, Braun DP, and Harris JE: Multiple concurrent suppressor cell mechanisms in human malignant disease. Blood 54(1):97A, 1979.

7. Braun DP, Cobleigh MA, and Harris JE: The rebound-overshoot phenomenon: A consequence of selective cytotoxic drug effect on immunoregulatory suppressor mechanisms. Proc Am Assoc Cancer Res 21:373, 1980.

8. Braun DP, Rossof AH, and Harris JE: Relationship of monocyte percentage and monocyte suppressor activity to T lymphocyte function in solid tumor cancer patients. Proc Am Assoc Cancer Res 21:370, 1981.

9. Harris JE, and Braun DP: Effects of adjuvant chemotherapy for stage II breast cancer on immunoregulatory cell function. Proc Am Assoc Cancer Res 21:370, 1981.

10. Harris JE, and Braun DP: Effects of combination chemotherapy on immunoregulatory cell functions in solid tumor cancer patients: Correlation with rebound-overshoot recovery of immunity. Proc Am Assoc Cancer Res 21:183, 1981.

11. Braun DP, and Harris JE: Monocyte suppression and activation in solid tumor cancer patients. Proc Am Assoc Cancer Res 21:185, 1981.

12. Chiu KM, Braun DP, and Harris JE: Suppression of alloimmune response in normal individuals and cancer patients by indomethacin sensitive cells. Clin Res 29:735A, 1981.

13. DeBoer KP, Braun DP, and Harris JE: Adherent cell suppression of natural cytotoxicity in solid tumor patients. Clin Res 29:735A, 1981.

14. Rubenstein M, Braun DP, and Harris JE: Abnormal prostaglandin metabolism in cancer patient monocytes. Clin Res 29:737A, 1981.

15. Braun DP, DeBoer KP, and Harris JE: Relationship of monocyte prostaglandin metabolism and monocyte indomethacin sensitive immunoregulatory function in peripheral blood mononuclear cells of disseminated solid tumor cancer patients. Blood 58:70a, 1981.

16. Harris JE, Braun DP, DeBoer KP, and Vahey AL: Enumeration with monoclonal antibodies of leukocyte subsets in peripheral blood of solid tumor cancer patients. Blood 58:83a, 1981.

17. Harris JE, Vahey AL, and Braun DP: Quantitation with monoclonal antibodies of leukocyte subsets in peripheral blood mononuclear cells of solid tumor cancer patients. Proc Am Soc Clin Oncol 1:42, 1982.

18. Chiu KM, Droin J, Harris JE, and Braun DP: The immunological response of Wistar rats to the C-6 glioma cell line. Proc Am Assoc Cancer Res 23:241, 1982.

19. Harris JE, and Braun DP: Suppressor cell development predicts for tumor recurrence in surgically resected lung cancer. Proc Am Assoc Cancer Res 23:250, 1982.

20. Penn RD, Showel J, Harris JE, Braun DP, and Wolter J: Immunoregulatory cell studies in patients with gliomas. Proc Am Assoc Cancer Res 23:251, 1982.

21. Braun DP, and Harris JE: Relationship of arachidonic acid metabolism and indomethacin sensitive immunoregulatory function in peripheral blood mononuclear cells of disseminated solid tumor cancer patients. Proc Am Assoc Cancer Res 23:262, 1982.

22. Harris ZL, Braun DP, and Harris JE: Interferon therapy causes impaired lymphoproliferation by prostaglandin mediated mechanisms. Clin Res 30(1):747A, 1982.

23. Harris JE, and Braun DP: The effect of interferon therapy on immunoregulatory cells in the peripheral blood of patients with renal cell carcinoma. Immunobiol 163:255, 1982.

24. Braun DP, and Harris JE: Effect of chemotherapy on immunoregulation in solid tumor cancer patients: Correlation with changes in monocyte PGE synthesis and lymphocyte PGE sensitivity. Proc Am Assoc Cancer Res 24:215, 1983.

25. Harris JE, Harris ZL, and Braun DP: Depression of monocyte induced lymphoproliferative responses in peripheral blood mononuclear cells (PBMC) from renal cell carcinoma patients treated with human leukocyte interferon (IFN-α). Proc Am Assoc Cancer Res 24:205, 1983.

26. Chiu KM, McPherson LH, Harris JE, Slayton RE, and Braun DP: The separation of cytotoxic human peripheral blood monocytes into phagocytic and nonphagocytic subsets by centrifugal elutriation. Proc Am Assoc Cancer Res 24:215, 1983.

27. Braun DP, Harris JE, and Taylor SG IV: Effect of chemotherapy on monoclonal antibody defined leukocyte subsets in solid tumor cancer patients. Proc Am Soc Clin Oncol 2:56, 1983.

28. Penn RD, Braun DP, Harris JE, and Rossof AH: Modulation of natural killer cell function in brain tumor patients by glass adherent suppressor cells. Proc Am Soc Clin Oncol 3:57, 1984.

29. Braun DP, Harris JE, and Taylor SG III: Effect of chemotherapy on monoclonal antibody defined leukocyte subsets in solid tumor cancer patients. Proc Am Assoc Cancer Res 25:234, 1984.

30. Braun DP, and Harris JE: Chemotherapeutic modulation of NK function in peripheral blood mononuclear cells of cancer patients. Proc Am Assoc Cancer Res 25:234, 1983.

31. Braun DP, Wolter J, and Harris JE: NK function and immunoregulation in peripheral blood mononuclear cells (PBMC) from sarcoma patients treated with fibroblast interferon. Proc Am Soc Clin Oncol 3:56, 1984.

32. Janus T, Slayton WB, Harris JE, Rossof AH, and Braun DP: Modulation of lymphocyte responsiveness to phytohemagglutinin (PHA) by micromolecular fibrinogen degradation products (FDP) due to induction of monocyte suppressor cells. Proc Am Assoc Cancer Res 25:243, 1984.

33. Harris JE, Wiederhold MD, Slayton J, and Braun DP: Abnormal arachidonate conversion to prostaglandins, leukotrienes and eicosatetraenoic acids in cancer patient monocytes. Blood 64:190a, 1984.

34. Wiederhold MD, Harris JE, and Braun DP: Modulation of arachidonate incorporation into macrophage membrane phospholipids by interferon. Fed Proc 44:951, 1985.

35. Braun DP, Von Roenn J, and Harris JE: Modification of chemotherapy induced immunosuppression in lung cancer patients with piroxicam. Proc Am Soc Clin Oncol 4:223, 1985.

36. Taylor SG IV, Harris JE, and Braun DP: Potentiation of T cell immunity in head and neck cancer patients with nonsteroidal antiinflammatory agents. Proc Am Assoc Cancer Res 26:278, 1985.

37. Leslie WT, Harris JE, Slayton RE, Wiederhold MD, Slayton JM, and Braun DP: Modulation of arachidonic acid metabolism in monocytes by α- and γ-interferon. Proc Am Assoc Cancer Res 26:279, 1985.

38. Harris JE, and Braun DP: Abnormal monocyte function in patients with Kaposi's sarcoma. Proc Am Assoc Cancer Res 26:286, 1985.

39. Anderson KM, Harris JE, Saffold P, and Braun DP: Modulation of cyclic nucleotide responses in lymphocytes by prostaglandin and interleukin 2. Proc Am Assoc Cancer Res 26:303, 1985.

40. Wiederhold MD, Slayton JM, Harris JE, Anderson KM, and Braun DP: Stimulation of phospholipase activity in U937 cells following treatment with α- and γ-interferon. Proc Am Assoc Cancer Res 26:307, 1985.

41. Williams GS, Harris JE, and Braun DP: The role of arachidonic acid metabolism in spontaneous cytotoxicity and immunoregulation of subsets of human peripheral blood monocytes. Proc Am Assoc Cancer Res 26:307, 1985.

42. Braun DP, Harris JE, Slayton JM, and Wiederhold MD: Altered arachidonic acid metabolism in cancer patient monocytes. Proc Am Assoc Cancer Res 26:307, 1985.

43. Harris JE, Wiederhold MD, Slayton JM, and Braun DP: Arachidonate incorporation and release from the membrane phospholipids of cancer patient monocytes. J Leukocyte Biol 38:133, 1985.

44. Williams GS, Harris JE, and Braun DP: Differences in accessory cell function of subsets of human peripheral blood monocytes. J Leukocyte Biol 38:180, 1985.

45. Harris JE, Janus TJ, and Braun DP: Fibrinogen degradation products (FDP) induce normal monocytes to become suppressor cells by modifying arachidonic acid metablism. Blood 66:87a, 1985.

46. Ondrey F, Wiederhold MD, Anderson KM, Braun DP, and Harris JE: Early PMA-induced morphologic changes, release of arachidonic acid, and synthesis of eicosanoids in U937 cells: Proc Am Assoc Cancer Res 27:58, 1986.

47. Leslie WT, Taylor SG IV, Harris JE, and Braun DP: Immune studies in renal cell carcinoma (RCC) patients treated with interferon. Proc Am Assoc Cancer Res 27:321, 1986.

48. Harris JE, and Braun DP: Modification of chemotherapy induced immunosuppression by the nonsteroidal antiinflammatory drug (NSAID), piroxicam. Proc Am Assoc Cancer Res 27:328, 1986.

49. Braun DP, Roseman D, and Harris JE: Enhanced interleukin 1 (IL 1) synthesis by monocytes from colon cancer patients. Proc Am Assoc Cancer Res 27:345, 1986.

50. Ebie N, Harris JE, Anderson KM, Wolter J, and Braun DP: Cyclic nucleotide responses in peripheral blood mononuclear cells of cancer patients following stimulation with interleukin 2 (IL 2) or prostaglandin E (PGE). Proc Am Assoc Cancer Res 27:345, 1986.

51. Wiederhold MD, Anderson KM, Ondrey F, Braun DP, and Harris JE: Analysis of monocyte cellular phospholipid fatty acid composition in patients with renal cell carcinoma with and without lymphoblastoid interferon (Hu IFN Ly) therapy. Proc Am Assoc Cancer Res 27:349, 1986.

52. Williams GS, Harris JE, Rossof AH, and Braun DP: Arachidonic acid (AA) metabolism differences in subsets of human peripheral blood monocytes separated by elutriation (CCE). Proc Am Assoc Cancer Res 27:350, 1986.

53. Harris JE, Landay AL, Falk L, Paul D, and Braun DP: Monocyte functional disturbances in asymptomatic homosexual males (AHM), AIDS-related complex syndrome (ARCS) patients, and aquired immune deficienty patients (AIDS). Blood 68:127a, 1986.

54. Landay AL, Kessler H, Braun DP, Harris JE, Paul D, and Falk L: Isolation of human immunodeficiency virus (HIV) from peripheral blood monocytes of subjects at risk for AIDS. Blood 68:129a, 1986.

55. Siebert P, Wiederhold MD, Anderson KM, Ondrey F, Harris JE, and Braun DP: Morphologic changes and alterations in eicosanoid metabolism induced in U937 cells by γ-interferon. Proc Am Assoc Cancer Res 28:339, #1341, 1987.

56. Conlon K, Harris JE, and Braun DP: Modulation of lymphokine activated killer (LAK) cell induction with eicosanoid synthesis inhibitors. Proc Am Assoc Cancer Res 28:367, #1455, 1987.

57. Chu EB, Potempa LA, Harris JE, Gewurz H, and Braun DP: Modulation of monocyte cytotoxicity with urea-chelated and acid-modified C-reactive protein (CRP). Proc Am Assoc Cancer Res 28:344, #1362, 1987.

58. Braun DP, Staren ED, and Harris JE: Lymphokine activated killer cell (LAK) induction in tumor infiltrating leukocytes (TIL) and peripheral blood mononuclear cells (PBMC) from colon cancer patients. Proc Am Assoc Cancer Res 28:371, #1472, 1987.

59. Potempa LA, Gewurz H, Harris JE, and Braun DP: Modulation of monocyte function by modified antigenically distinct forms of C-reactive protein (CRP). Proc Am Assoc Cancer Res 28:344, #1363, 1987.

60. Harris JE, Taylor SG IV, and Braun DP: Modulation of lymphokine activated killer (LAK) cell induction following cytotoxic chemotherapy. Proc Am Assoc Cancer Res 28:372, #1475, 1987.

61. Creekmore SP, Harris JE, Ellis TM, Braun DP, McMannis JD, Cohen II, Bhoopalam N, Jassak PF, Cahill MA, Canzoneri CL, and Fisher RI: Phase I/II trial of recombinant interleukin 2 by 24 hour continuous infusion. An Illinois Cancer Council Trial. Proc Am Soc Clin Oncol 6:244, #960, 1987.

62. Harris JE, Landay AL, Kessler H, Falk L, Paul D, and Braun DP: Abnormal indomethacin sensitive suppressor cell (ISSC) function is associated with deficient lymphokine activated killer (LAK) cell induction in patients infected with the human immunodeficiency virus (HIV). Proc Am Soc Clin Oncol 6:3, #11, 1987.

63. Ellis T, Braun DP, Creekmore SP, Bhoopalam N, Harris JE, and Fisher RI: Appearance and phenotypic characterization of circulating Leu 19^+ cells in patients receiving recombinant IL 2. Proc Am Assoc Cancer Res 28:373, #1480, 1987.

64. McMannis JD, Braun DP, Fisher RI, Creekmore SP, Harris JE, and Ellis TM: Demonstration of circulating lymphokine activated killer (LAK) cells in patients receiving interleukin 2 (IL 2). Proc Am Assoc Cancer Res 28:372, #1474, 1987.

65. Landay AL, Harris JE, Falk L, Paul D, Kessler H, and Braun DP: Abnormal lymphokine activated killer cell (LAK) induction in peripheral blood mononuclear cells (PBMC) in patients infected with the human immunodeficiency virus (HIV). Proc 3rd International Conference on AIDS, 1987.

66. Levy RA, Harris JE, Bagdade JD, and Braun DP: Marine lipids suppress monocyte mediated cytotoxicity in women with insulin dependent diabetes mellitus. Clin Res 35(6):826A, 1987.

67. Braun DP, and Harris JE: Improved response to monocyte activators in the peripheral blood monocytes of patients with solid tumors following cytotoxic chemotherapy. Proc Am Assoc Cancer Res 29:373, #1484, 1988.

68. Chu EB, Potempa LA, Harris JE, Gewurz H, and Braun DP: Reversal of Neo-C-reactive protein mediated suppression of monocyte cytotoxicity by modulation of arachidonate metabolism. Proc Am Assoc Cancer Res 29:371, #1477, 1988.

69. Staren ED, Braun DP, Harris JE, and Economou SG: Lymphokine activated killer cell induction in tumor infiltrating leukocytes and peripheral blood mononuclear cells from colon cancer patients. Proc Am Assoc Cancer Res 29:406, #1617, 1988.

70. Taylor SG IV, Harris JE, and Braun DP: Modulation of monocyte cytotoxicity by arachidonic acid metabolic inhibitors in the peripheral blood monocytes of patients with solid tumors. Proc Am Assoc Cancer Res 29:371, #1478, 1988.

71. Staren ED, Harris JE, and Braun DP: Lymphokine activated killer (LAK) cell induction in the peripheral blood mononuclear cells (PBMC) of colon cancer patients. Proc Am Soc Clin Oncol 7:98, #373, 1988.

72. Braun DP, Siziopikou KP, and Harris JE: Modulation of monocyte cytotoxicity in response to granulocyte/macrophage colony stimulating factor (GM-CSF) and γ-interferon (γ-IFN) by arachidonic acid metabolic inhibitors. Blood 72:142a, 1988.

73. Gaynor ER, Ellis TM, Harris JE, Braun DP, Tuteur D, Creekmore S, and Fisher RI: Adjuvant immunotherapy using intermittent 24 hour infusion of recombinant interleukin 2 (rIL 2) in patients with surgically resected colon carcinoma at high risk for recurrence. Proc Am Soc Clin Oncol 8:190, #739, 1989.

74. Braun DP, Harris JE, Landay AL, and Kessler H: Impaired development of monocyte tumor cytotoxicity in response to γ-interferon (γ-IFN) and granulocyte/macrophage colony stimulating factor (GM-CSF) in the peripheral blood monocytes of asymptomatic human immunodeficiency virus (HIV) infected patients. Potentiation of responsiveness by indomethacin. Proc Am Soc Clin Oncol 8:1, #4, 1989.

75. Staren ED, Braun DP, Harris JE, Bines SD, and Economou SG: Characterization of interleukin 2 (IL 2) activated killer cells in tumor infiltrating leukocytes (TIL) from colon cancer patients. Proc Am Soc Clin Oncol 8:103, #398, 1989.

76. Siziopikou KP, Casey LC, Harris JE, and Braun DP: Imparied development of tumoricidal function in the alveolar macrophages (AM) of patients with lung cancer. Proc Am Assoc Cancer Res 30:334, #1323, 1989.

77. Chu EB, Potempa LA, Gewurz H, Harris JE, and Braun DP: Modulation of the development of tumoricidal function in response to macrophage activators by a modified form of the C-reactive protein. Proc Am Assoc Cancer Res 30:333, #1322, 1989.

78. Braun DP, Siziopikou KP, and Harris JE: Modulation of the *in vitro* development of tumoricidal function in response to combinations of macrophage activators in the peripheral blood monocytes (PBM) of cancer patients (pts). Proc Am Assoc Cancer Res 30:334, #1324.

79. Taylor SG IV, Harlan S, McHale M, Rudolph A, Braun DP, and Harris JE: Phase I dose escalation of TNF/IL 2 combination therapy in human malignancies. Proc Am Assoc Cancer Res 30:405, #1609, 1989.

80. Staren ED, Economou SG, Harris JE, Bines S, and Braun DP: Tumor induced modulation of lymphokine activated killer cell induction in tumor infiltrating leukocytes. Proc Am Assoc Cancer Res 31:238, #1405, 1990.

81. Braun DP, Siziopikou KP, Casey L, and Harris JE: Augmentation of impaired tumoricidal function in alveolar macrophages from lung cancer patients by cocultivation with allogeneic lymphocytes. Proc Am Assoc Cancer Res 31:244, #1443, 1990.

82. Siziopikou KP, Casey L, Harris JE, and Braun DP: Tumor necrosis factor secretion, interleukin 1 secretion, and the development of cytotoxicity in alveolar macrophages from lung cancer patients. Proc Am Assoc Cancer Res 31:244, #1442, 1990.

83. Harris, JE, Siziopikou KP, Ahn M, Wilbanks GD, Jordan E and Braun DP: The induction of tumoricidal function in regional versus systemic macrophages from patients with solid tumors. Proc Am Assoc Clin Oncol, 10:214, 1991.

84. Chu EB, Gewurz H, Harris JE and Braun DP: Arachidonic acid metabolism in macrophages treated with a modified form of the C-reactive protein. Proc Am Assoc Cancer Res, 32:232, 1991.

85. Braun DP, Siziopikou KP, Ahn M and Harris JE: Induction of tumoricidal function in macrophages from cancer patients by anti-CD3 monoclonal antibodies. Proc Am Assoc Cancer Res, 32:232, 1991.

86. Harris JE, Siziopikou KP, Casey LM and Braun DP: Sensitivity of alveolar macrophages and peripheral blood monocytes to modulation of arachidonic acid metabolism. Proc Am Assoc Cancer Res, 32:233, 1991.

87. Harris JE, Ahn M and Braun DP: Differential sensitivity of peripheral blood monocytes (PBM), alveolar macrophages (AM) and peritoneal macrophages (PM) from cancer patients to modulation of arachidonic acid (AA) metabolism. Blood 78:1072, 1991.

88. Harris JE and Braun DP: Natural Killer (NK) cell function in renal cell cancer patients (RCC pts): Identification of a subset responsive to immunological manipulation. Proc. Am. Soc. Clin. Oncol. 11:257, 1992.

89. Braun DP, Ahn M, and Harris JE: Sensitivity of human macrophages from different anatomical sites to arachidonic acid metabolism. Proc. Am. Can. Res. 33:305, 1992.

90. Ahn M, Harris JE, Wilbanks GW, and Braun DP: Induction of tumoricidal function in macrophages by anti-CD3 monoclonal antibodies. Proc. Am. Assoc. Can. Res. 33:305, 1992.

91. Ahn M, Plate JMD, Harris JE, and Braun DP: Downregulation of Interleukin 6 in alveolar macrophages of lung cancer patients facilitates the development of tumoricidal function. Blood 80:103a, 1992.

92. Braun DP, Ahn M, Harris JE, Wilbanks G, and Plate J: Altered synthesis of cytokine messenger RNA (m RNA) in peripheral blood monocytes and peritoneal macrophages from patients with gynecological malignancies. Proc. Am. Soc. Clin. Oncol. 12:299, 1993.

93. Ahn M, Plate J, Harris JE, and Braun DP: Allogeneic lymphocytes stimulate macrophage tumoricidal function in association with IL-6 suppression. Proc. Am. Assoc. Cancer Res. 34:445, 1993.

94. Harris JE, Ahn M, and Braun DP: Interleukin-12 (IL-12) is superior to gamma interferon (IFNγ) plus endotoxin and to Monocyte Colony Stimulating Factor (M-CSF) in stimulating tumoricidal function in alveolar macrophages (AM) from patients with non-small cell lung cancer. Blood, 82:509a, 1993.

95. Taylor SG IV, Braun DP, Coon JS, Hutchinson JC, Panje WR, Caldarelli DD, Shott S and Preisler HD. Augmentation of autologous tumoricidal function in peripheral blood monocytes from head and neck cancer patients by treatment with indomethacin. Proceedings American Association for Cancer Research 35: 480, 1994.

96. Braun DP, Coon JS, Taylor SG IV, Hutchinson JC, Panje WR, Caldarelli DD, Shott S and Preisler HD. Discordance of leukocyte-mediated tumoricidal function in the blood and leukocyte infiltration in tumor tissues. Proceedings American Association for Cancer Research 35: 494, 1994.

97. Effect of endometriosis on the production of cytokines by peripheral blood monocytes. D.P. Braun, N. Rana, C. Rotman, H.M. Gebel and W.P. Dmowski. Proc. Amer. Fertil. Soc. 50: s20, 1994.

98. Effect of danazol (DZ) or gonadotoprin releasing hormone agonist (GnRH-A) therapy on the production of cytokines by peripheral blood monocytes (PBM) from patients with endometrosis (ENDO). D.P. Braun, N. Rana, C. Rotman, H.M. Gebel and W.P. Dmowski. Proc. Amer. Fertil. Soc. 50: 149, 1994.

99. Braun DP, Tereshkova N, Ortegel J and Preisler HD. Cytotoxic function of peripheral blood blast cells from patients with acute myelogenous leukemia. Proceedings American Association for Cancer Research 36: 473, 1995.

100. Braun, DP, Taylor, SGT IV, Coon, J, Griem, K. LaFollette, S. Hutchinson, J, Kotelnikov, V, and Preisler, HP. Biological and Immunological Differences in Primary and Recurrent Head and Neck Cancer. Proc. Amer.Soc.of Clin. Oncol. 14:22,1995.

101. Taylor SGT IV, Braun DP, Kotelnikov VM, Wood NB, Mundle S, Coon J, Raza A, LaFollette S, Griem K, Hutchinson J, Cladarelli D, Panje W, Friedman M, Preisler H. Tumor proliferative and immunologic alterations during combined chemotherapy/radiation in Head and Neck Cancer. Proc. ASCO. 15:, 315, 1996.

102. Braun DP, House R, Gebel HG, Rana N, and Dmowski WP. Differential Sensitivity of endometrial cells from uterus and peritoneal implants to macrophage-mediated lysis in patients with endometriosis. Proceedings of the Vth World Congress on Endometriosis. Yokohama, Japan. October, 1996.

103. Braun DP, House R, Gebel HG, Rana N, and Dmowski WP. Macrophage functional changes in women with unexplained infertility identifies a subset of patients with subclinical endometriosis. Proceedings of the Vth World Congress on Endometriosis. Yokohama, Japan. October, 1996.

104. DmowskiWP, Rana N, Braun DP, Gebel HG, and House R. Is there a pathophysiological basis for generalized symptoms in women with endometriosis? Proceedings of the Vth World Congress on Endometriosis. Yokohama, Japan. October, 1996.

105. Braun DP, Gebel HG, Rana N, and Dmowski WP. Cytolysis of endometrial cells (EC) by autologous peripheral blood monocytes (PBM) and peritoneal macrophages (PM) in women with endometriosis (Endo). Proceedings of the 52nd Annual Meeting, American Society of Reproductive Medicine, 1996.

106. Braun DP, Harris JE, Gebel H, Plate J, Deziel D, Millikan K, Doolas A and Prinz R. Immunologic Evaluation of pancreatic cancer patients. Proc. Amer. Assoc. Cancer Res. 38:487, 1997.

107. Ortegel J, Staren E, Faber P, Warren W, and Braun D. Cytokine biosynthesis by tumor infiltrating T cells in non-small cell lung cancers. Proc. Amer. Assoc. Cancer Res. 38:630, 1997.

108. Braun, DP, Dmowski, WP. Stimulation of eutopic and ectopic endoetrail cell proliferation by autologous peritoneal fluids with endometriosis is due to tumor necrosis factor-alpha (TNFα). Oral Presentation, ASRM/CFAS '99 Conjoint Annual Meeting, Ontaior, Canada, September 25-30,1999.

109. Ding, J, Shen, J, Braun, DP, Rana; N, Fernandez, BB, Dmowski, WP. Apoptosis in Endometrial Glandular and Stromal Cells in Women with and without Endometriosis. Oral Presentation, ASRM/CFAS '99 Conjoint Annual Meeting, Ontario, Canada, September 25-30, 1999.

110. Shen, J., Ding, J, Braun, DP, Rana, N, Dowbrowski, A, Dmowski, WP. Endometrial Macrophages May Be Involved in the Regulation of Endometrial Apoptosis. Poster Presentation, ASRM/CFAS '99 Conjoint Annual meeting, Ontario, Canada, September 25-30, 1999.

111. Ding, J, Shen, M, Gogacz, M, Braun, DP, Rana, N., Dmowski, WP. Endometrial Apoptosis Is Inhibited In Vitro by TNFα in Women with Endometriosis and Is Stimulated by TNFα in Healthy Women. Journal of the Society for Gynecologic Investigation, 2000.

112. Braun, DP, Ding, J, Shen, M, Gogacz, M., Dmowski, WP. Modulation of Endometrial Cell Apoptosis and Proliferation by Tumor Necrosis Factor in Women with Endometriosis. Journal of the Society for Gynecologic Investigation, 2000.

113. Ding, J, Gogacz, Shen, M, Braun, DP, Rana, N, Dmowski. Expression of TNFα Receptor Type-I Gene in Endometrial Cells from Women with and without Endometriosis. Submitted to American Society For Reproductive Medicine, 56th Annual Meeting, October 21-25, 2000, San Diego, California.

UNIVERSITY INSTRUCTION:

Course Director

Principles of Immunology. University of Illinois, Dept. of Microbiology, 1977.

Biology of Cancer. College of Nursing, Rush Presbyterian St. Luke's Medical Center, 1982.

Tumor Immunology. Department of Immunology, College of Medicine, Rush Presbyterian St. Luke's Medical Center,1985.

The Basic Science of Oncology. Rush Cancer Institute. 1993-1995.

Lecturer

Basic and Clinical Immunology, Department of Immunology/Microbiology
Cellular Immunology, Department of Immunology/Microbiology

Tumor Immunology, Department of Nursing
Pathophysiology of Malignant Disease, Department of Pathology
Medical Oncology Didactic Course

Organizing

Medical Oncology Didactic Lecture Series
Medical Oncology Research Seminar Series
Oncology Fellow Basic Sciences Journal Club

Advisory

Graduate Students Matriculated (5)
Dissertation Advisory Committees (18)
As Chairman (2)
As Advisor (5)
As Member (11)
Other Department (2)
Other University (5; 4, University of Illinois, 1 Notre Dame)

UNIVERSITY COMMITTEES:

Student Judiciary Review
Academic Freedom, Chairman, 1994-1996
CED Review of Rush Medical College
Radiation Oncology Search Committee
General Surgery Search Committee
Department of Dermatology Search Committee
Department of OB/Gyn Search Committee
Faculty Council, 1996-1999.
Task Force on Interaction with Biomedical Industry, Chairman.
Academic Council

INVITED PRESENTATIONS:

1. Transfer of Plasmacytoma Immunity with Immune RNA Extracts from Tumor-bearing Balb/C mice. American Dental Association, Chicago, IL, 1977.

2. Principles of Cancer Immunology. University of Indiana School of Medicine. Gary, Indiana, 1978.

3. Synergy between Cytotoxic Chemotherapy and Anti-Tumor Immunity in Solid Tumor Cancer Patients. Department of Pathology, University of Illinois Medical Center, Chicago, IL, 1979.

4. Selective Effects of Cytotoxic Chemotherapy on Suppressor Cells in Cancer Patients. Illinois Cancer Council, Chicago, IL, 1980.

5. Immunoregulatory Cell Function and Impaired Immunity in Patients with Gliomas. Department of Neurosurgery, Cook County Hospital, 1983.

6. Effect of Interferon Therapy on Prostaglandin-producing Suppressor Cells in Renal Cell Cancer Patients. Burroughs Wellcome, Research Triangle Park, North Carolina, 1984.

7. Changes in Prostaglandin Metabolism in Monocytes from Interferon-treated Cancer Patients.Department of Microbiology, University of South Florida, Tampa, Florida, 1985.

8. Potentiation of Immunity in Chemotherapy-Treated Cancer Patients. Northwestern School of Medicine, Cancer Center, Chicago, IL, 1985.

9. Prostaglandin-Producing Suppressor Cells in Cancer Patients and AIDS Patients. Department of Pathology, Loyola University School of Medicine, Chicago, IL. 1986.

10. Synergy between Chemotherapy and Immunity in Solid Tumor Cancer Patients. Roswell Park Memorial Institute, Grace Cancer Center, Buffalo, New York, 1986.

11. Modification of the Effects of Cancer Chemotherapy on Immune Responses in Lung Cancer Patients by Treatment with Piroxicam. Pfiser Pharmaceuticals, New Orleans, Louisiana, 1986.

12. Enhancement of Deficient Cellular Immunity in Head and Neck Cancer Patients Treated with Piroxicam. Pfizer Laboratories, Annaheim, California, 1987.

13. Modulation of Immunity in Cancer Patients by Prostaglandin Antagonists. Second International Conference on Immunity to Cancer. Williamsburg, Virginia, 1987.

14. Potential for Combining Cytotoxic Chemotherapy and Biological Response Modifiers in Cancer Patients. Illinois Cancer Council, Chicago, IL, 1988.

15. Monocyte Immunoregulatory Cell Function in HIV-Infected Patients. American Red Cross, Chicago, IL, 1988.

16. Lymphokine Activated Killer Cell Function in Tumor Infiltrating Leukocytes from Colon Cancer Patients. Illinois Cancer Council Symposium on Biological Response Modifiers, Chicago, IL, 1988.

17. Principles of Cancer Immunology. Department of Surgery, Grant Hospital Chicago, IL, 1990.

18. Immune Function in Cancer Patients and the Effects of Chemotherapy. Lederle Laboratories Symposium. Chicago, IL, 1990.

19. Danazol Effects on Peritoneal Macrophage Function in Patients with Endometriosis. Sterling International. New York, NY, 1991.

20. Peritoneal Macrophage Function in Endometriosis. Chicago Association of Reproductive Endocrinology. Chicago, IL, 1991.

21. The Biology and Immunology of Cancer. American College of Surgeons, Chicago, IL, 1991.

22. Immunotherapy of Cancer Patients, Baxter/Bartels Oncology Focus Meeting. Chicago, IL, 1992.

23. Systemic and Local Tumor Immunity in Patients with Solid Tumors. Abbott Laboratories, Abbott Park, IL, 1992.

24. Modulation of Endometrial Cell Proliferation by Monocytes in Patients with Endometriosis. Chicago Association of Reproductive Endocrincology. Chicago, IL, 1992.

25. Modulation of Tumoricidal Function in Tumor-Associated Macrophages from Solid Tumor Patients. Section of Hematology/Oncology, University of Chicago Chicago, IL, 1992.

26. Stimulation of Endometrial Cell Proliferation by Monocytes in Endometriosis Patients. Adeza Biomedical Corp. Sunnyvale, CA. 1993.

27. Cytokine Synthesis by Circulating Monocytes in Patients with Endometriosis. Chicago Association of Reproductive Endocrinology. Chicago, IL, 1994.

28. Effects of Virulizin on Macrophage Functions of Cancer Patients. Hoffman LaRoche, Nutley, NJ, 1994.

29. Macrophage Functional Changes in Unexplained Infertility. Chicago Association of Reproductive Endocrinology. Chicago, IL, 1995.

30. Differential Sensitivity of Ectopic and Eutopic Endometrial Cells to Macrophage-Mediated Cytolysis in Women with Endometriosis. Chicago Association of Reproductive Endocrinology. Chicago, IL, 1996.

31. Effects of Virulizin on Macrophage Functions of Cancer Patients and Women with Endometriosis. Schering Plough, NJ, 1996.

32. Chemotherapy-induced immune modulation in cancer patients. National Cancer Institute of Japan-Tokyo Japan, January, 1997.

33. Macrophage functions in cancer and endometriosis and its modulation by virulizin. Pharmacia. Milano, Italy, February, 1997.

34. Strategies for immune stimulation in cancer patients. Connaught Laboratories, Toronto, Canada, October, 1997.

Exhibit B

1515

Tumor inflammatory response induced by immunization with autologous melanoma cells conjugated to dinitrophenol(DNP). D. Berd, M.J. Mastrangelo, C. Green, C. Clark, and E. Hart. Thomas Jefferson University, Philadelphia, PA 19107.

Treatment of melanoma patients with an autologous vaccine preceded by low dose cyclophosphamide (CY) induces delayed-type hypersensitivity (DTH) to melanoma cells, and in some cases, regression of metastatic tumors. Now, we are attempting to increase the efficiency of the process by immunizing with tumor cells conjugated to the hapten, DNP. Patients with metastatic melanoma were sensitized to DNP by topical application of dinitrochlorobenzene (DNCB). Two weeks later, they were injected with a vaccine consisting of 10-25x10(6) autologous, irradiated melanoma cells conjugated to DNP and mixed with BCG. CY 300 mg/M^2 IV was given 3 days before DNCB or vaccine. Of 4 patients evaluable so far, 3 have developed a striking inflammatory response in tumor masses after 2 vaccine treatments.(8 weeks). Patient #1 developed erythema and swelling in the >50 large (1-3 cm) dermal metastases on her leg and lower abdomen, followed by ulceration and drainage of necrotic material, and some are beginning to regress. Biopsy showed infiltration with CD4+ and CD8+ T lymphocytes. Patient #2 developed erythema and swelling in the skin of her lower abdomen and groin overlying large (8 cm) nodal masses. These have not yet regressed, but have changed in consistency from rock-hard to fluctuant. Patient #3 exhibited moderate erythema in the skin overlying subcutaneous metastases. All 3 patients have developed DTH to both DNCB and to DNP-conjugated autologous lymphocytes. Although these results are preliminary, they suggest that this new strategy may represent a significant advance in the immunotherapy of human melanoma.

1516

Inhibition of Tumor-Induced Suppressor T Lymphocyte (Ts) Activity by Murine Interferon Beta (IFN-B). Deepak M. Sahasrabudhe, University of Rochester Cancer Center, Rochester, NY, 14642

In some tumor models inhibition of Ts-activity is a prerequisite to successful immunotherapy. Based on our data in the DNFB model (J Exp Med 166:1573, 1987) the effect of IFN-B on P815 mastocytoma-induced Ts-activity was evaluated.

In this model, concomitant antitumor immunity (Tc) peaks by Day 10 and is down regulated by Ts by Day 15. Cytotoxicity generated after a mixed lymphocyte tumor culture (MLTC) correlates with in vivo immunity and suppression of cytotoxicity correlates with in vivo Ts-activity.

Tumors were initiated by injecting 2 x 10^6 P815 cells subcutaneously on Day 1. IFN-B (10U, 1000U, 5000U) or buffer were injected i.v. every other day x 5 doses starting on Day 5. On Day 16, MLTC's were set up. Five days later a cytotoxicity assay was performed against ^{51}Cr labelled P815 cells. % specific lysis is shown. Numbers in parenthesis represent the dose of IFN-B.

E:T	Tc	Tc + Naive	Ts	Tc +Ts	Ts (10)	Tc +Ts (10)	Ts (1000)	Tc +Ts (1000)	Ts (5000)	Tc +Ts (5000)
50:1	88	81	0	19	6	22	23	20	81	84
25:1	84	76	0	12	2	21	1	21	63	75
12:1	78	79	2	15	3	24	6	23	58	81
6:1	70	69	1	7	0	9	0	20	38	64
3:1	56	55	0	8	1	13	0	12	21	48

Treatment with IFN-B 5000U every other day x 6 doses abrogated Ts-activity without adversely affecting cytotoxicity. IFN-B may be a useful adjunct in the immunotherapy of selected tumors.

1517

Anti-idiotype monoclonal antibody immunization therapy of cutaneous T cell lymphoma. Chatterjee, M., Foon, K., Seong, B.K., Barcos, M. and Kohler, H., Roswell Park Mem. Inst., Buffalo, NY 14263, and UCSD, San Diego, CA 92161.

Cutaneous T cell lymphoma (CTCL) is an indolent non-Hodgkin's lymphoma which is not cured by standard therapies once it reaches advanced stage. A novel approach to therapy is to use internal image anti-idiotype (Id) mAb as an antigen (Ag) substitute for the induction of immunity. We have generated anti-Id mAb (Ab2) binding to a hybridoma SN2 (Ab1), which recognizes a unique glycoprotein, gp37, expressed by a subset of human leukemic T cells (J. Immunol. 139:1354, 1987). At least 2 of these Ab2 may indeed carry the internal image of the gp37 Ag (J. Immunol. 141:1398, 1988). Recently, we investigated the distribution of gp37 Ag by a sensitive immunoperoxidase staining method using mAb SN2. SN2 had a high specificity for T-leukemia/lymphoma cells and did not react with any normal adult tissues tested including thymus, lymphocytes, bone marrow cells, spleen, liver, kidney, lung, brain, heart, etc. CTCL cells from 5/6 out of 6 patients were strongly positive for gp37 Ag with intense surface membrane staining. The binding of radiolabeled SN2 to CTCL cells was studied for inhibition in the presence of the anti-Id mAbs 4EA2 and 4DC6 which mimic the gp37 Ag. Both clones inhibited the binding 100% and 80% respectively at a concentration of 50 ng. We also generated a murine Ab3 mAb (anti-anti-Id) by immunizing mice with the anti-Id mAb (Ab2). This Ab3 mAb reacts with CTCL cells in an identical fashion as the original Ab1 (SN2). Collectively, these data suggest that Ab2 4EA2 and 4DC6 may be useful for active immunotherapy of CTCL patients. We also plan to study the CTCL patients in a phase I clinical trial to determine the effects of this type of therapy on various components of the immune system (both humoral and cellular) and try to identify the criteria to select patients who may benefit from anti-idiotype vaccine therapy.

1518

Syngeneic murine monoclonal antiidiotypes bearing the internal image of a human breast cancer associated antigen. J. Schmitz and H. Ozer. The Dept. of Microbiology, S.U.N.Y. at Buffalo, Buffalo, NY 14214 and the Division of Medical Oncology, The Univ. of North Carolina at Chapel Hill, Chapel Hill, NC 27599.

According to Jerne's network theory, some antiidiotypes (Ab2) mimic external antigens recognized by specific antibodies (Ab1) and may be used in place of antigen for immunization. The murine monoclonal antibody F36/22 (IgG3, κ), specific for ductal carcinoma antigen (DCA) was used to generate syngeneic monoclonal antiidiotypes bearing the internal image of DCA. Female BALB/c mice were inoculated intraperitoneally every other week with 100 μg of F36/22 coupled to keyhole limpet hemocyanin; the first time in complete Freund's adjuvant and subsequently in incomplete adjuvant. Splenic lymphocytes were fused with the murine cell line P3X63 Ag8.653 3 days after the fourth immunization using 50% polyethylene glycol (m. w. 2000). Two hybrids, MTO-1 and MTO-2, were selected based on the ability of culture supernatants to bind to F36/22 but not to the control antibody 2A31F6 (IgG3, κ) in an enzyme linked immunosorbent assay (ELISA) and cloned by limiting dilution. Paratope specificity of Ab2 was demonstrated in two ELISA assays. First, the binding of labeled F36/22 to DCA was inhibited 100% and 75% by 1.6 μg of MTO-2 and MTO-1 respectively. Second, the binding of labeled Ab2 to Ab1 was inhibited by purified DCA. MTO-1 neither enhances nor inhibits the binding of labeled MTO-2 to Ab1 although in the presence of MTO-2, binding of labeled MTO-1 is enhanced by 100% indicating that these Ab2 recognize distinct idiotopes. Rabbits immunized bi-weekly with MTO-1 or MTO-2 developed antibodies that bound specifically to DCA demonstrating that MTO-1 and MTO-2 bear the internal image of DCA. These data suggest that MTO-1 and MTO-2 could potentially be utilized to immunize high risk patients against progression or development of DCA positive tumors.

Exhibit C

TUMOR VACCINATION

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An autologous whole-cell vaccine has been shown to induce DHR to the whole-cell component, as well as significant regression of metastasis in patients with metastatic malignant melanoma.

Tumor vaccination is an active, specific immunotherapy for malignant disease. It may be defined as "the administration of tumor cells, modified tumor cells, or tumor-cell surface-membrane preparations to stimulate or to augment various components of antitumor immunity to induce tumor regression or to prolong tumor remission achieved by conventional therapy."[1]

Vaccines also may be considered a type of biologic response-modifier therapy. The approach is based on the belief that the host is capable of mounting an effective immune response against tumors if appropriately stimulated, a belief that was first advanced around the turn of the century.[2] The concept of immunologic surveillance, which evolved some 40 to 50 years later, suggested that the human host was capable, under certain circumstances, of rejecting a tumor essentially in the same manner as a homograft was rejected.[3]

The first attempt to vaccinate humans against cancer was undertaken in 1902.[4] In this initial attempt, fluid was extracted from tumors in patients with advanced disease.[5] Over the next 50 years, a great variety of tumor cell preparations obtained from autologous or allogeneic tumors were used, generally to treat patients with advanced disease.

Fresh interest in the clinical potential of tumor vaccination was stimulated in the 1950s and 1960s by experimental studies conducted in syngeneic rodents. These demonstrated unequivocally that chemically induced and virally induced tumors had both shared and uniquely individual tumor-specific transplantation antigens (TSTAs).

Humoral and cellular immune responses were shown to exist in patients with cancer; these were found to be directed against tumor-associated antigens (TAAs) rather than against TSTA. Also, TAAs were found on embryonic cells and tumor cells. The "unique antigens" on human tumor cells appear to result from tumor cell dedifferentiation for display of a partial embryonic-cell-membrane antigenic profile. Other TAAs arise as a consequence of the modification of normal "self" antigens producing an "altered self" phenotype.

TABLE 1

Antitumor Immune Mechanisms

- Activated macrophage cytotoxicity
- Cytotoxic T cells
- Natural killer (NK) cells
- Lymphokine-activated killer cells
- Humoral antibodies (complement dependent)
- Antibody-dependent cellular cytotoxicity (complement-independent, macrophage, neutrophil or NK-cell-dependent)

MANY UNRESOLVED CLINICAL ISSUES

The principal human immune responses to tumor antigens are listed in Table 1. It has not been definitively established which of these immune responses, alone or in combination, are the most important in a host response to cancer, nor is it clear which should be targeted for stimulation with tumor vaccination. There are many unresolved issues pertaining to the actual vaccine formulation that need to be addressed; some of these depend on whether the vaccine formulation is based on intact tumor cells (Table 2) or tumor cell extracts or products (Table 3).

In addition to vaccine formulation, several other questions remain:

- Should cellular extracts or whole cells be mixed with immunomodulating adjuvants

to increase tumor vaccine immunogenicity?

- Should tumor vaccines with or without adjuvants be used alone or in combination with cytotoxic drugs that can modulate or suppress undesirable immune responses?
- Should cytokines be used to augment immune responses to a vaccine?
- Is there a place for tumor vaccination in patients with advanced cancer?
- What is the appropriate dose, schedule, and route of administration for effective tumor vaccination?
- What measurement or surrogate biologic end point can be used to assess the biologic effectiveness of the vaccine?
- Will the immune response against TAA produced by a human tumor vaccine be selective and specific for tumor cells or will autoimmune reactions against normal cells be a possible toxicity associated with vaccination?

Advances in molecular genetics and the availability of monoclonal antibody reagents now make it possible to purify cells and cell components with defined and unique antigenic characteristics for use in human tumor vaccines. However, a number of the promising clinical trials of tumor vaccination conducted in the 1970s and 1980s used relatively simple and empiric methods of tumor vaccination preparation.

In one study, surgically resected stage I and II lung cancer patients were treated with a vaccine prepared from allogeneic tumor cells.[6] Cell membranes from viable tumor cells were subjected to low-frequency sonication and the soluble material separated with Sephadex G-200. Polyacrylamide gel electrophoresis was used to purify protein band material, which could elicit delayed hypersensitivity reactions (DHRs) in lung cancer patients. This material was administered intracutaneously in combination with Freund's complete adjuvant (FCA) in a series of three injections at monthly intervals beginning about 1 month after surgery.

Pilot studies suggested that this form of therapy delayed or prevented tumor recurrence. The approach was tested in a large multicenter clinical trial, which found no difference in survival between control patients and patients treated with FCA alone or with FCA and tumor antigen.[6] No autoimmune toxicity was noted during the course of these studies. Peripheral blood monocytes producing excessive amounts of prostaglandins appeared in the circulation prior to clinical relapse in patients who failed in all three arms of the study.

TABLE 2

Critical Issues for Whole-Tumor-Cell Vaccines

Should autologous or allogeneic cells be employed?
Should cells be obtained from fresh surgical specimens or from tissue cell lines?
Should cells first be irradiated to maintain their membrane integrity but prevent their proliferation?
How can the reproducibility of vaccine preparation be assured?
How can whole tumor cells be used that are gene modified for the following phenotypic changes (individual or in combination) to enhance immunogenicity: (1) expression of HLA class I or II antigens and/or adhesion molecules; (2) secretion of immunomodulatory stimulating cytokines, such as interleukin-2 or tumor necrosis factor; and (3) secretion of chemotactic cytokines?

Further analysis of this clinical trial, however, suggests that a survival benefit may have been obtained in the fraction of patients in whom careful attention was paid to thorough homogenization of tumor antigen in the FCA. Vaccinated long-term survivors also may have developed more intense DHRs to tumor antigen. The methods employed in this study, although important and innovative, need to overcome the problematic nature of the technique's purification process and reproducibility (Table 3) before wider application in humans is feasible.

A tumor vaccination study in patients with surgically resected Dukes B_2 through C_2 colorectal cancer was conducted, based on rigorously evaluated preclinical experimental animal data in which requirements for effective immunotherapy were established.[7] An elegant series of studies of a guinea pig line-10 hepatocarcinoma model showed convincingly that bacillus Calmette-Guérin (BCG) admixed with syngeneic tumor cells could induce sufficient systemic immunity to elimi-

The concept of immunologic surveillance... suggested that the human host was capable under certain circumstances of rejecting a tumor essentially in the same manner as a homograft was rejected.

Humoral and cellular immune responses were shown to exist in patients with cancer; these were found to be directed against tumor-associated antigens....

nate a limited metastatic disease burden.[8] These studies controlled for variables such as the number and viability of tumor cells, and ratio of viable BCG organisms to tumor cells. In the pilots that evolved from these trials, patients were randomized to a control arm or were vaccinated with their own tumor cells, obtained from surgical specimens at the time of operation and cryopreserved until thawed and irradiated prior to use.[9] Treated patients underwent a schedule of three intradermal vaccine treatments weekly beginning 4 to 5 weeks after tumor resection.

The first two vaccine preparations consisted of irradiated cells and BCG; the third vaccine preparation was composed of irradiated tumor cells alone. Vaccinated patients developed augmented DHR to their autologous tumor cells with greater frequency than nonvaccinated patients. A DHR increase to autologous normal intestinal mucosa cells was not seen. An Eastern Cooperative Oncology Group trial is now evaluating this approach for surgically resected Dukes B_2 and B_3 patients.

FIRST TRIALS WITH HUMAN TUMOR VACCINES

These studies, the first truly large, randomized, controlled, multi-institutional clinical trials of human tumor vaccines for solid tumors, are examples of the use of whole cells and cell extracts for human tumor-vaccine preparation. In each case, an adjuvant substance was added to enhance the immunogenicity of the vaccine.

An autologous whole-cell vaccine has been shown to induce DHR to the whole-cell component, as well as significant regression of metastasis in patients with metastatic malignant melanoma.[10] Patients received cyclophosphamide before the vaccine in an attempt to modulate the activity of suppressor T-lymphocytes. The vaccine was prepared by methods similar to those previously described[7] and combined with BCG.

Other investigators have also used cyclophosphamide to inhibit suppressor T-lymphocyte activity prior to the administration of a malignant melanoma vaccine.[11] The vaccine preparation consisted of mechanically disrupted allogeneic tumor cells from melanoma cell lines. The concentration of vaccine was standardized in the preparation through measurements of a melanoma-associated antigen.

Measurement was performed by binding inhibition enzyme immunoassays using a monoclonal antibody. The vaccine was given subcutaneously with an adjuvant consisting of detoxified endotoxin (monophosphoryl lipid A) mycobacterial cell wall skeleton and squaPane oil. In this trial, regressions of disease were seen in patients with metastatic disease.

VIRAL ONCOLYSATES FOR HUMAN TUMOR VACCINATION

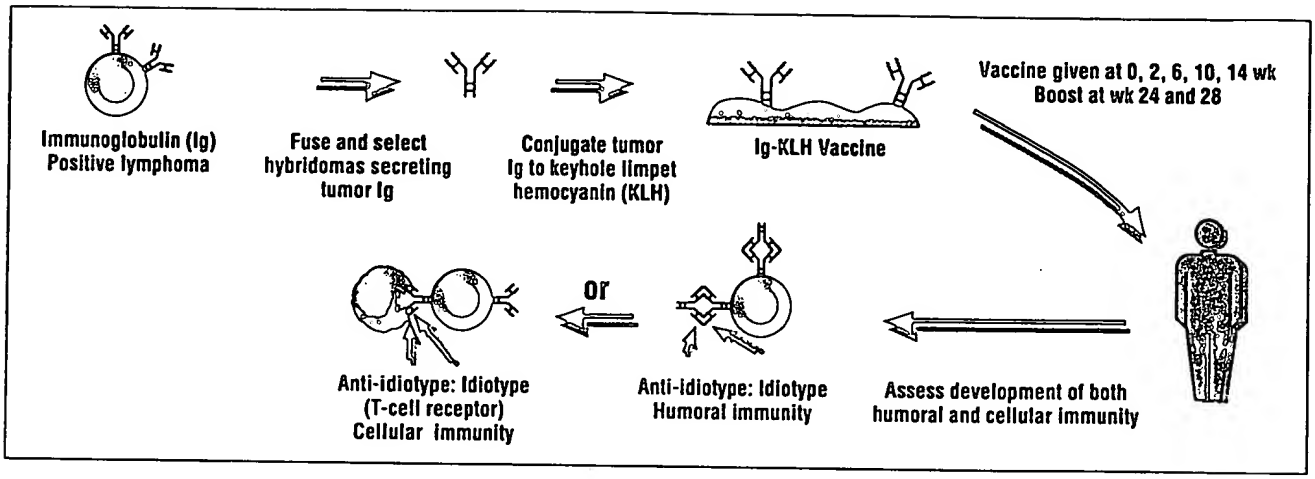
The use of viral oncolysates for human tumor vaccination combines the potential immunogenic benefit of whole cells with the value of cell extracts.[12] Viral oncolysates are homogenates of virus-infected cells. The virus in the mixture is believed to have an adjuvant rather than an antigenic role. Allogeneic and autologous viral oncolysates have been used in human immunotherapy. Influenza virus and vaccinia virus have been most frequently used in the preparation of viral oncolysates since the first report of this procedure in 1974.[13]

Pilot studies have suggested a protective or therapeutic benefit for viral oncolysates in gynecologic cancer, melanoma, and sarcoma. However, these reports must be considered anecdotal until larger randomized investigations are conducted.

TABLE 3

Critical Issues for Tumor-Cell-Extract Vaccines

What method of antigen extraction should be employed?
Should material be derived from a single source or should pooled material from a number of sources be used?
What methods are to be used to identify material in cellular extracts that will produce the most effective stimulation of antitumor immune responses?
Should extracted material be separated from HLA antigens that are present on both normal and malignant cells?
How can reproducibility of vaccine preparation be assured?



FIGURE

Strategy for idiotype vaccination. Source: Adapted from Kwak LW, Campbell MJ, Czerwinski DK, Hart S, Miller R, Levy R. Induction of immune responses in patients with B cell lymphoma against surface immunoglobulin idiotype expressed by their tumors. *N Engl J Med.* 1992; 327:1209-1215.

It may be possible to vaccinate against some human cancers by immunizing against those few viruses presently known to be associated with cancer in humans. Hepatitis B virus infection is associated with the development of primary hepatocellular cancer. Immunizing against this virus will prevent its hepatic damage and may reduce the incidence of associated cancer.[14]

Finally, one of the most innovative approaches to tumor vaccination that has been developed relies on the use of idiotypic molecules that reiterate the molecular configuration of tumor-associated antigens. This approach is based on principles that predict that the variable regions of immunoglobulins and T-cell receptors that are responsible for antigen recognition are themselves capable of provoking both B-cell and T-cell immunity.[15]

These concepts led other investigators to vaccinate patients who have B-cell lymphoma with the autologous immunoglobulins from each patient's tumor following cytotoxic chemotherapy[16] (Figure). Vaccinated patients developed either humoral immunity, cellular immunity, or both; in the two patients with measurable disease, complete tumor regression was observed. These preliminary results demonstrate the feasibility of idiotypic vaccination for B-cell and T-cell malignant diseases and suggest that similar approaches might also be developed for nonlymphoreticular malignancies.

REFERENCES

1. Hersh EM, Marligit GM, Gutteman GM, and Richman SP. Immunotherapy of human cancer. In: Becker FF, ed. *Cancer: A Comprehensive Treatise.* New York, NY: Plenum Press; 1977; 425.
2. Ehrlich P. (1909) The collected papers of Paul Ehrlich. In: Himmelweit F, ed. *Immunology and Cancer Research.* London: Pergamon Press; 1957:550.
3. Burnet M. *Cellular Immunology.* Cambridge, England: Cambridge University Press; 1969.
4. Von Leydon E, Blumenthal F. Vorlaufige mitteilunger über einige ergebenisse der krebsforschung auf der I. Medizinischen Klinik Dtsch. *Med Wochenschr.* 1902;28:637-638.
5. Southam CM. Applications of immunology to clinical cancer: past attempts and failure possibilities. *Can Res.* 1961;21:1302-1316.
6. Stewart THM, Shelley WE, Willan AR, Hollinshead AC. An evaluation of the role of tumor-specific antigens. In: Mountain C, ed. *Lung Cancer: Current Status and Prospect for the Future.* Houston, Texas: University of Texas Press; 1986: 351.
7. Hoover HC, Hanna MG. Immunotherapy by active specific immunization: clinical applications. In: DeVita VT, Hellman S, Rosenberg SA, eds. *Biological Therapy of Cancer.* Philadelphia, Pa: JB Lippincott Company, 1991.
8. Hoover HC, Peters LC, Brandhorst JS, et al. Therapy of spontaneous metastases with an autologous tumor vaccine in a guinea pig model. *J Surg Res.* 1981;30:409-415.
9. Hoover HC, Surdyke MG; Dangel RB, et al. Prospectively randomized trial of adjuvant active-specific immunotherapy for human colorectal cancer. *Cancer.* 1985;55:1236-1243.
10. Berd D, Maguire HC Jr, Mastrangelo M. Induction of cell-mediated immunity to autologous melanoma cells and regression of metastases after treatment with a melanoma cell vaccine preceded by cyclophosphamide. *Can Res.* 1986;46:2572-2577.
11. Mitchell MS, Harel W, Kempf RA, et al. Active-specific immunotherapy for melanoma. *J Clin Oncol.* 1990;8:856-869.
12. Ioannides CG, Platsoncas CD, Patenia R, et al. T-Cell functions in ovarian cancer patients treated with viral oncolysates. Increased helper activity to immunoglobulins production. *Anticancer Research.* 1990;10:645-654.
13. Sinkovics JG, Williams DE, Campos LT, et al. Intensification of immune reactions of patients to cultured sarcoma cells: attempts at monitored immunotherapy. *Semin Oncol.* 1974;1:351-365.
14. Stevenson FK. Tumor vaccine. *FASEB J.* 1991;5:2180-2257.
15. Schwartz RS. Therapeutic clonotypic vaccines. *N Engl J Med.* 1992; 327:1236-1237.
16. Kwak LW, Campbell MJ, Czerwinski DK, et al. Induction of immune responses in patients with B-cell lymphoma against the surface immunoglobulin idiotype expressed by their tumors. *N Engl J Med.* 1992;327:1209-1215.

Exhibit D

[CANCER RESEARCH 38, 204-209, January ...8]

Immunotherapy of Established Micrometastases with *Bacillus Calmette-Guérin* Tumor Cell Vaccine[1]

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ABSTRACT

We evaluated the use of *Bacillus Calmette-Guérin* admixed with tumor cells as a vaccine to induce systemic tumor immunity for therapy of subclinical (micrometastatic) disease. In several experiments inbred strain 2 guinea pigs were given i.v. injections of either 10^4, 10^5, or 10^6 syngeneic L10 hepatocarcinoma cells, and initial vaccinations were administered either 1 or 4 days after tumor inoculation. Variables in vaccine preparation, such as ratio of viable *Bacillus Calmette-Guérin* organisms to tumor cells, procedures for freezing the tumor cells, X-ray treatment of tumor cells, and vaccination regimen were evaluated. The studies demonstrated that under defined conditions nontumorigenic vaccines of *Bacillus Calmette-Guérin* and tumor cells can cure the majority of animals of otherwise lethal visceral micrometastases.

INTRODUCTION

The strategy of immunotherapy for cancer in experimental animal models and humans is limited by many factors including the stage, type, and location of the tumor; the level of antigenicity of the tumor cells; and the status of the host immune response. Clinical immunotherapy has been proceeding with relatively limited guidance from experimental animal models. Of the several approaches to immunotherapy of localized tumor and/or disseminated minimal residual tumor, immune potentiation by microbial agents has received the greatest attention. The most encouraging experimental and clinical data to date have resulted from protocols consisting of bacterial vaccines or nonspecific immunostimulants, primarily *Mycobacterium bovis* strain BCG,[2] administered i.t. (17, 18, 21) or systemically either alone (7, 8, 16) or admixed with tumor cells in the form of a vaccine (22, 23). One impetus for the use of BCG in immunotherapy has been the development of an experimental system that meets some of the requirements of a model to study an established tumor with regional lymph node metastasis (19). It has been demonstrated that regression of transplanted syngeneic hepatocarcinomas growing in the skin of inbred strain 2 guinea pigs and elimination of regional lymph node metastases are achieved in the majority of animals after i.t. injection of viable BCG (12, 26). This particular aspect of immunotherapy in the guinea pig model, although intriguing, is very limited with respect to the type, stage, and location of the tumor as well as with respect to the route of administration of BCG. Nevertheless, the initial studies established 1 fact that has broad implications. During BCG-mediated tumor regression and elimination of regional lymph node metastases, there is the development of systemic cell-mediated tumor immunity demonstrated by rapid rejection of a second tumor challenge several weeks after BCG treatment (11, 25, 27). This is a very important aspect of the model since it is known that, at the tumor stage when BCG administration is optimally effective, surgical excision of the tumor and regional lymph node would also be curative. However, no significant development of tumor immunity is achieved with surgery alone.

We recently demonstrated the effectiveness of tumor immunity induced by i.t. injections to eliminate artificially produced distant tumor foci (9, 10). This aspect of the BCG therapy model becomes important when one considers that adjuvant immunotherapy has been primarily tested in cancers for which control of primary tumors is available with surgery, radiotherapy, and/or chemotherapy, but where there is a substantial rate of relapse. Recurrence is usually thought to be due to a small number of residual tumor cells. Adjuvant immunotherapy is intended to eradicate the residual tumor cells by enhancing immunological mechanisms. However, based on all that we have learned, the translation to humans of the results of i.t. BCG injection in the guinea pig model would require careful attention to certain aspects of the treatment. These include tumor stage, dose, injection, route, regimen, and source of BCG. This is not always possible in human cancer, for which immunotherapy is often used for advanced cancer after other forms of treatment have failed. In addition, this model is inappropriate for cases in which i.t. injections are not possible.

An important advance in this guinea pig immunotherapy model would be to achieve effective systemic tumor immunity without the i.t. injection of BCG. We have approached this problem by systematically evaluating the ability of vaccines of BCG admixed with tumor cells to eliminate a disseminated tumor burden. Although previous attempts at BCG-tumor cell vaccine immunotherapy both in inbred guinea pigs (3) and in humans (see Ref. 20 for review) have been limited and somewhat discouraging, relatively little has been done to determine the optimal conditions for vaccination. Here we investigate a number of variables such as the ratio of viable BCG organisms to tumor cells, the freezing procedures, the X-ray treatment of cells, and the vaccination regimen. Although these factors cannot possibly be investigated systematically in humans for ethical reasons, they can be studied in the guinea pig model. Our studies demonstrate that, under defined conditions,

[1] Research sponsored by the National Cancer Institute under Contract NO1-CO-25423 with Litton Bionetics, Inc.

[2] The abbreviations used are: BCG, *Bacillus Calmette-Guérin*; i.t., intratumoral; i.l., intralesional; L10, line 10 hepatocarcinoma cells; HBSS, Hanks' balanced salt solution; i.d., intradermal; PPD, purified protein derivative of *Bacillus Calmette-Guérin*.

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nontumorigenic vaccines of BCG and tumor cells can cure the majority of animals with lethal disseminated tumors established as visceral micrometastases.

MATERIALS AND METHODS

Animals. Inbred male Sewall Wright strain 2 guinea pigs were obtained from the Frederick Cancer Research Center Animal Breeding Section. These guinea pigs were shown to be histocompatible by skin grafting. They were housed 6 to 10 per cage and fed Wayne guinea pig chow and kale; they weighed 400 to 500 g at the beginning of the experiments.

Tumors. Induction of primary hepatocarcinomas in strain 2 guinea pigs after they were fed the water-soluble carcinogen, diethylnitrosamine, was described previously (19). The antigenic and biological properties of the transplantable ascites tumors developed from the primary hepatocarcinomas have also been described (28).

Ascites hepatocarcinoma cells, L10, were harvested and washed 3 times in HBSS and diluted to desired concentrations. One-ml doses of L10, ranging from 10^4 to 10^6 cells/dose, were injected into the dorsal vein of the penis, producing artificial vascular metastasis. Injections of 10^4 cells resulted in the death of approximately 70 to 80% of the animals, whereas 10^5 and 10^6 cells were fatal to all animals. The times of death varied as a function of dose, and all animals died as a result of metastasis to the lung, mediastinal lymph nodes, and hilar lymph nodes with concurrent visceral metastases.

BCG. *M. bovis* strain BCG (Phipps strain TNC 1029) was obtained from the Trudeau Institute (Saranac Lake, N. Y.). Preparations of BCG, stored at −70°, were rapidly thawed in a 37° water bath and diluted to proper concentrations.

Vaccine Preparation. The L10 tumor was maintained by i.p. passage in guinea pigs. Ascites cell preparations were removed and washed in HBSS. The L10 cells used in vaccine preparation were either fresh or frozen and thawed.

In preparation for freezing, the cells were concentrated and suspended in an equal volume of chilled 15% dimethyl sulfoxide plus 10% fetal calf serum-HBSS solution. The final suspension was 2 to 6 × 10^7 cells/ml. Two-ml aliquots of the L10 cell suspension were frozen at controlled rates in a Linde BF4 Biological Freezer at −1°/min to the critical freezing point, flash-frozen through the heat of fusion, and continued at −1°/min to a final temperature of −60°. The rate of freezing was monitored on a Honeywell Electronic III. The vials were stored in liquid nitrogen. The rationale for this method of freezing has been described in detail elsewhere (14, 15). The vials were rapidly thawed in a 37° water bath. Frozen-thawed cells were slowly diluted to 50 ml in HBSS, washed once, and resuspended in preparation for X-irradiation. Suspensions of fresh and frozen-thawed cells were X-irradiated in 50-ml beakers on ice. X-irradiation was performed with a Phillips MG 301 X-irradiation unit at 500 R/min. A total X-irradiation dose of 20,000 R was achieved. Cell viability counts were performed with the use of the trypan blue dye exclusion test, and viability after irradiation of either fresh or frozen-thawed cells was generally 90%, with less than 10% variation between the fresh or frozen-thawed cells.

BCG (10^9 organisms/ml) was added in equal volume to viable L10 (10^8 cells/ml) for a vaccine ratio of 10:1. A vaccination consisted of an i.d. injection of 0.2 ml. For ratios of 1:10, BCG (10^9 organisms/ml) was diluted 1:100 in HBSS, and aliquots were mixed with 10^6 viable L10 cells/ml. These vaccinations also consisted of an i.d. injection of 0.2 ml. All vaccinations were performed less that 1 hr after the BCG-tumor cell mixtures were prepared.

In preliminary vaccination experiments, the L10 cells were irradiated with 12,000 R; however, we noticed that, although this irradiated cell preparation was not tumorigenic when admixed with BCG, it was tumorigenic when administered i.d. in the absence of BCG. We were concerned that any growth of 12,000-R X-irradiated L10 cells in the skin might preempt developing tumor immunity and thus render the treatment ineffective against disseminated tumor. Therefore, 20,000-R X-irradiation was used in all subsequent experiments with L10 cells in BCG-tumor cell vaccines. Animals were given i.v. injections, in the dorsal vein of the penis, of either 10^4, 10^5, or 10^6 L10 cells in 1-ml volumes. All vaccinations were given i.d., beginning in the upper right dorsal quadrant. Successive vaccinations were given in different sites or i.l. in the previous vaccination site. Vaccinations were performed either 1 and 7 days or 4 and 10 days after i.v. L10 injection.

RESULTS

An i.v. dose of 10^4 L10 tumor cells does not lead to the death of all guinea pigs. Approximately 25% of the animals will survive clean injections where leakage did not occur to the regional site. This inoculum is the optimal dose for assessing the influence of the nonspecific side effects of vaccination on tumor cell arrest, the extravasation and establishment in organs, and the immunologically specific effects of the vaccine. Thus, at this initial tumor cell dose of 10^4, vaccinations were performed at either 1 and 7 or 4 and 10 days after i.v. injections of L10.

Several modes of vaccination as well as 2 ratios of viable BCG to tumor cells were tested in guinea pigs given i.v. injections of 10^4 L10 cells. The BCG-tumor cell ratios were 10^8 BCG or 10^6 BCG admixed with 10^7 L10. These were administered as either a single vaccination, a single injection of BCG-L10 vaccine followed 6 days later by an i.l. injection of L10 into the previous vaccination site, a single injection of BCG-L10 vaccine followed 6 days later by an injection of L10 alone on the opposite side, or 2 separate injections of BCG-L10 vaccine. Also, the efficacy of frozen L10 cells was compared to that of fresh L10 cells. The results are shown in Table 1.

Compared to the untreated tumor-bearing guinea pigs, no significant difference in survival was detected in animals treated with 2 i.d. injections of BCG or tumor cells alone, regardless of whether the initial treatment was performed 1 or 4 days after i.v. injection of L10.

Single BCG + L10 vaccinations at ratios of 1:10 or 10:1 did not confer significantly greater protection than did vaccinations of BCG alone, tumor cells alone, or nontreated controls. Furthermore, these 2 BCG:L10 ratios could not be associated with significant differences in survival of animals given i.v. injections of 10^4 tumor cells, regardless

Table 1

Survival of guinea pigs given i.v. injections of 10^4 syngeneic L10 hepatocarcinoma cells

This experiment was terminated at 280 days after tumor injection; all nontreated controls died by 120 days. Significance of differences in survival was calculated by the Fisher 2-tailed exact test

Treatment[a]	No. of survivors/total no. of animals/group at following vaccination times after i.v. injection of tumor	
	Days 1 and 7	Days 4 and 10
None	3/12	
(10^6 BCG) (10^6 BCG)	3/12	3/10
(10^7 L10) (10^7 L10)	2/10	
(10^6 BCG + 10^7 L10)[b]	4/10	4/10
(10^6 BCG + 10^7 L10)[b]	2/10	
(10^6 BCG + 10^7 L10) (10^7 L10 i.l.)	8/10	8/10
(10^6 BCG + 10^7 L10) (10^7 L10 i.l.)	9/10	9/10
(10^6 BCG + 10^7 FL10[c]) (10^7 FL10 i.l.)	8/10	
(10^6 BCG + 10^7 L10) (10^7 L10)	10/10	10/10
(10^6 BCG + 10^7 L10) (10^7 L10)	10/10	9/10
(10^5 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	10/10	10/10
(10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	9/10	10/10

[a] Treatments were administered 6 days apart on opposite sides as described in "Materials and Methods."
[b] Vaccinations were administered as single sequential injections.
[c] FL10, frozen-thawed L10.

of the vaccination schedule. Compared to those animals that received single vaccinations of BCG + L10, BCG, or tumor cells alone and compared to the nontreated controls, significant differences in survival were achieved in tumor-bearing guinea pigs that received the second vaccination of either L10 i.l. ($p < 0.03$), L10 on the opposite side ($p < 0.01$), or BCG-L10 mixture ($p < 0.01$). From 80 to 100% of the animals survived in these treatment groups, regardless of whether the initial vaccine was administered 1 or 4 days after i.v. L10 injection. No significant differences in efficacy were detected between fresh L10 cells and frozen L10 cells.

At 280 days, representative groups of the survivors either were tested for tumor immunity by measurement of rejection of i.d. challenge of 10^6 L10 cells or were killed and autopsied for gross and histological examination for residual tumor. None of the animals autopsied had any evidence of residual tumor. Tumor challenge groups varied in their ability to reject contralateral challenge as a function of treatment. All nontreated controls or groups that had been treated with BCG or tumor cells alone failed to reject contralateral challenge, indicating that these animals were not tumor immune at 280 days after treatment. Seventy to 90% of the survivors in the various multiple vaccination groups rejected contralateral challenge; however, no significant difference in tumor immunity, as measured by contralateral challenge, could be detected among these treatment groups. These data demonstrate that animals that survived after treatment with ineffective modes of vaccination were not tumor immune, whereas significant protection as well as long-term tumor immunity was conferred on those animals that received efficacious modes of vaccination.

Injections of 10^5 or 10^6 syngeneic L10 cells i.v. are routinely fatal in strain 2 guinea pigs. Vaccinations of BCG alone or tumor cell alone conferred no protection in these tumor-bearing guinea pigs when the animals were given vaccinations 1 and 7 days after i.v. tumor inoculation (Table 2). Survival in all treatment groups was a function of the BCG:L10 cell ratio. Without exception, in guinea pigs given 10^5 or 10^6 cells i.v., a vaccine containing BCG:L10 cells in a ratio of 10:1 yielded significant protection, whereas a ratio of 1:10 was ineffective. Thus, the ratio of viable BCG organisms to tumor cells is a critical factor in the efficacy of the vaccine, and a large amount of BCG is beneficial in the initial vaccination. No significant difference in protection could be detected when the group that received a single BCG + L10 vaccination (10:1) was compared to a similar treatment group that received a second i.l. L10 injection. In contrast, survival was achieved in those animals that received a second injection of L10 alone or BCG + L10 on the opposite side ($p < 0.02$ or $p < 0.01$, respectively). In 2 groups of animals given i.v. injections of 10^5 L10, no significant difference in protection was detected when frozen-thawed L10 was used in the vaccine in place of the fresh L10.

One important consideration was whether BCG-immune guinea pigs could generate effective tumor immunity after

Table 2

Survival of guinea pigs given i.v. injections of 10^5 or 10^6 syngeneic L10 hepatocarcinoma cells

These experiments were terminated at 240 days after tumor injection. All nontreated controls in the 10^5 group died by 95 days, and all nontreated controls in the 10^6 group died by 77 days. Significance of differences in survival was calculated by the Fisher 2-tailed exact test.

Treatment[a]	No. of survivors/total no. of animals/group at following i.v. tumor cell dose	
	10^5	10^6
None	0/10	0/10
(10^6 BCG) (10^6 BCG)	0/10	0/10
(10^7 L10) (10^7 L10)	0/10	0/10
(10^6 BCG + 10^7 L10)[b]	1/10	0/10
(10^6 BCG + 10^7 L10)[b]	2/10	0/10
(10^6 BCG + 10^7 L10) (10^7 L10 i.l.)	1/10	
(10^6 BCG + 10^7 L10) (10^7 L10 i.l.)	5/10	
(10^6 BCG + 10^7 FL10[c]) (10^7 L10 FL10 i.l.)	5/10	
(10^6 BCG + 10^7 L10) (10^7 L10)	1/10	0/10
(10^6 BCG + 10^7 L10) (10^7 L10)	8/10	3/10
(10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	1/10	1/10
(10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	9/10	8/10
(10^6 BCG + 10^7 FL10) (10^6 BCG + 10^7 FL10)	9/10	

[a] Treatments were administered 6 days apart on opposite sides as described in "Materials and Methods."
[b] Vaccinations were administered as single sequential injections.
[c] FL10, frozen-thawed L10.

BCG + L10 vaccination. Normal guinea pigs as well as guinea pigs previously immunized to BCG and shown to be PPD positive by skin testing were given i.v. injections of 10^5 L10 cells. In this particular experiment vaccinations were performed 4 and 10 days after i.v. tumor inoculation. Two modes of vaccination, BCG + L10 (10:1) at Days 4 and 10 and BCG + L10 (10:1) at Day 4 followed by i.l. L10 at Day 10, were compared in PPD-positive and PPD-negative guinea pigs. Regardless of whether or not the animals were PPD positive, the 2 modes of vaccination conferred significant protection ($p < 0.01$) and did not differ significantly (Table 3).

We next investigated whether multiple BCG + L10 vaccinations at either the 10:1 or 1:10 ratios would improve survival in comparison to single or sequential vaccinations. Guinea pigs were given i.v. injections of 10^6 L10 cells and vaccinated on either 1 day, 1 and 7 days, or 1, 7, and 14 days after i.v. tumor inoculation. Treatments consisted of a single vaccination of BCG + L10 followed by L10 alone or by BCG + L10, 2 simultaneous BCG + L10 vaccinations followed by 2 simultaneous injections of L10 alone or of BCG + L10, or 3 sequential vaccinations of BCG + L10. The results are shown in Table 4.

Initial BCG + L10 vaccinations at a ratio of 1:10 were ineffective regardless of the vaccination schedule. Significant protection ($p < 0.01$) was achieved with all initial vaccinations at ratios of 10:1, but no significant increase in survival was achieved by multiple or sequential vaccinations.

DISCUSSION

Vaccines consisting of tumor cells admixed with BCG, under certain defined conditions, are effective in controlling and eliminating micrometastases in a syngeneic guinea pig tumor system, regardless of whether the animals are BCG immune or not. At the outset it should be stated that this experimental model has major limitations in that it is a transplantable tumor established for a short time in normal guinea pigs and in that the system out of necessity does not take into account such factors as individual variations between the biological behavior of tumors of other histological types and the limiting factors of the host. Thus, the model may be used to answer only specific questions fundamental to immunotherapy of micrometastasis.

Table 3

Survival of guinea pigs given i.v. injections of 10^3 syngeneic L10 hepatocarcinoma cells: effectiveness of vaccination in BCG-immune guinea pigs

Guinea pigs were given i.d. injections of 10^6 BCG and skin tested with PPD 21 days after immunization; 2 weeks later animals were given i.v. injections of 10^3 L10. The experiment was terminated 270 days after tumor injection, and all nontreated controls died by 128 days after tumor injection. Significance of differences in survival was calculated by the Fisher 2-tailed exact test.

PPD sensitivity	Treatment at 4 and 10 days	Survivors/total no. of animals/group
−	None	0
+	None	0
−	(10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	5/10
+	(10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	6/10
−	(10^6 BCG + 10^7 L10) (10^7 L10 i.l.)	2/10
+	(10^6 BCG + 10^7 L10) (10^7 L10 i.l.)	6/10

Table 4

Survival of guinea pigs given i.v. injections of 10^5 syngeneic L10 hepatocarcinoma cells: effect of multiple vaccinations

This experiment was evaluated at 120 days after i.v. tumor injection, and all animals in the nontreated or single vaccination control groups died by 70 days. Significance of differences in survival was calculated by the Fisher 2-tailed exact test.

Treatment[a]	Survivors/total no. of animals/group
None	0/13
(10^6 BCG + 10^7 L10)[b]	0/10
(10^6 BCG + 10^7 L10)[b]	0/10
(10^6 BCG + 10^7 L10) (10^7 L10)	0/10
2(10^6 BCG + 10^7 L10) 2(10^7 L10)[c]	1/9
(10^6 BCG + 10^7 L10) (10^7 L10)	3/10
2(10^6 BCG + 10^7 L10) 2(10^7 L10)	6/10
(10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	1/10
2(10^6 BCG + 10^7 L10) 2(10^6 BCG + 10^7 L10)	1/10
(10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	5/10
2(10^6 BCG + 10^7 L10) 2(10^6 BCG + 10^7 L10)	6/10
(10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	4/9

[a] Treatments were administered 6 days apart on opposite sides, as described in "Materials and Methods."
[b] Vaccinations were administered as single injections.
[c] Two simultaneous injections.

Under natural conditions the development of metastasis is dependent upon an interplay between properties of the host and properties of the tumor cells. The process is highly selective and represents the end point of several destructive events from which few tumor cells survive. Only a few tumor cells within the primary neoplasm may actually invade blood vessels, and of those even fewer will survive in the circulation. Similarly, not all malignant cells that survive transport are successfully arrested, undergo extravasation, etc. Also, tumor cells, in principle, could be susceptible to host immune and nonimmune defense mechanisms that could destroy malignant cells during any of the steps described above (5, 6).

Metastasis was artificially induced in guinea pigs by i.v. injection of L10, and treatment was not started until adequate time had elapsed to ensure extravasation and localization of tumor cells into the parenchyma of visceral organs. No significant difference in the effectiveness of vaccines was found when the treatment was started 1 or 4 days after tumor cell transplantation. It has previously been demonstrated with i.v. injection of B16 melanoma in mice (4) that, between 1 and 4 hr after i.v. transplantation, there is a 50% reduction in the number of arrested tumor cells in the lung, and at 24 hr only 2% of the cells are retained in the lung as a stable metastatic population. Thus, the results of any treatment administered prior to 24 hr after transplantation are impossible to interpret since beneficial effects could be due to prevention of metastasis rather than to treatment. In this study the lack of difference between

various effective vaccines when treatment was adminstered 1 or 4 days after transplantation suggests that a therapeutic effect was indeed achieved with these vaccinations.

It was clear from this study that, of the 2 basic BCG-tumor cell vaccines used, the preparation consisting of 10^6 viable BCG admixed with 10^7 tumor cells was more effective over a broad range of increasing initial tumor burdens than was that of 10^6 BCG admixed with 10^7 tumor cells. Although the latter was effective at initial tumor burdens at 10^4 L10, it was ineffective when the initial tumor cell inoculum was increased to 10^5 L10. Whether this 10:1 BCG:tumor cell ratio is critical or simply a function of total BCG cannot be determined from these studies. However, in a study of the effectiveness of BCG-tumor cell mixtures as vaccines against LSTRA murine leukemia (2), it was found that immunity was high (100%) if the BCG:LSTRA ratio was low (either 5×10^4:10^3 or 5×10^6:10^5) and that the proportion of immune mice was low (8%) if the BCG:LSTRA ratio was high (5×10^6:10^3).

Tumor cells that were frozen by an established procedure used for preservation of bone marrow in transplantation studies and assessed as an optimal procedure in several low-temperature biology studies (for review, see Ref. 24) were equally as effective in the vaccines as fresh tumor cells. This is contrary to the results of Bartlett *et al.* (1) who used glycerol as the freezing additive. Our cells were frozen in dimethyl sulfoxide and fetal calf serum. The striking difference, however, was the percentage of viability after freezing. Cell viability was approximately 90% after freeze-thawing. If for some reason viability fell below 80% during liquid nitrogen storage, the cells were discarded. In our opinion, the trypan blue exclusion test is a very conservative test of cell damage, and any trauma sufficient to render 20% of the cells sensitive to trypan blue may have severely damaged the remaining cells or altered their antigenicity. The viability of frozen cells in the experiments of Bartlett *et al.* ranged between 40 and 70% as determined by trypan blue exclusion. Thus, the difference in results with frozen L10 cells may be attributed to suboptimal *versus* optimal freezing conditions. Whether the 20,000-R X-irradiation dose of the tumor cells was an important aspect in the preparation of cells in vaccines is not known. However, studies are under way to test this point in this model since it is recognized that the use of 12,000 R is standard procedure for BCG-tumor cell vaccines in humans.

Of the 3 basic vaccination schedules tested, the 2 that were consistently effective for all tumor burdens were 10^6 BCG admixed with 10^7 L10 followed by 10^7 alone on the opposite side or 2 separate injections of 10^6 BCG admixed with 10^7 L10. The fact that BCG was not required in the second injection of the former schedule and the fact that multiple vaccinations did not improve therapy with respect to the latter schedule or with the less effective vaccine (10^6 BCG + 10^7 L10) suggest that the critical aspect of any of the vaccination schedules is the initial dose of BCG. Following the initial treatment with BCG, effective systemic tumor immunity can be achieved with i.d. injections of tumor cells alone at a different site.

A third vaccination schedule, which consisted of reintroduction of the tumor immunogen into the i.d. site previously injected with BCG + tumor cells, was effective at lower tumor burdens (10^4) but was less effective at initial tumor burdens of 10^5. This is in contrast to a similar schedule in which the second tumor cell vaccination was in a different i.d. site. The rationale for the second injection of tumor cells in the BCG-infected site was based on the study of Hawrylko (13), in which the dimensions of BCG-potentiated antitumor response against the murine mastocytoma P815 were investigated. One limitation that we found with this procedure was the difficulty in delivering the tumor cell inoculum in the previously infected dermal site. Early ulcerations of these injected dermal sites were limiting with respect to constant delivery of the tumor immunogen in the second injection.

In this study we have shown that visceral micrometastasis induced by i.v. injection of L10 can be cured by the systemic effect of a tumor cell-BCG vaccine. These results confirm our previous studies on the immunological susceptibility of i.v.-injected L10 cells (9, 10). We have now demonstrated that a nontumorigenic vaccine can affect immunotherapy. These results demonstrate that there is a critical dose for BCG in the initial vaccination but that BCG is not essential in the subsequent vaccination and that optimum therapy could be achieved with 2 vaccinations separated by a period of 6 days. Furthermore, the induced tumor immunity, which can cure the majority of guinea pigs with micrometastases is achieved by 2 vaccinations that require a total of 2×10^7 tumor cells (approximately 20 mg of tumor) administered over a period of 1 week. Also, the tumor cells, when frozen under established optimal conditions, maintain immunogenicity and can be used effectively in vaccines.

REFERENCES

1. Bartlett, G. L., Katsilas, D. C., Kreider, J. W., and Purnell, D. M. Immunogenicity of "Viable" Tumor Cells after Storage in Liquid Nitrogen. Cancer Immunol. Immunotherapy, *2*: 127–133, 1977.
2. Bartlett, G. L., Purnell, D. M., and Kreider, J. W. BCG Inhibition of Murine Leukemia: Local Suppression and Systemic Tumor Immunity Require Different Doses Science, *191*: 299–301, 1976.
3. Bartlett, G. L., and Zbar, B. Tumor-specific Vaccine Containing *Mycobacterium bovis* and Tumor Cells: Safety and Efficacy. J. Natl. Cancer Inst., *48*: 1709–1726, 1972.
4. Fidler, I. J. Metastasis: Quantitative Analysis of Distribution and Fate of Tumor Emboli Labeled with ^{125}I-5-Iodi-2'-deoxyuridine. J. Natl. Cancer Inst., *45*: 773–78, 1970.
5. Fidler, I. J. Patterns of Tumor Cell Arrest and Development. *In*: L. Weiss (ed.), Fundamental Aspects of Metastasis, pp. 276–289, Amsterdam: Elsevier/North-Holland, Inc., 1976.
6. Fidler, I. J., and Nicolson, G. L. Organ Selectivity for Implantation and Growth of B16 Melanoma Variant Tumor Lines. J. Natl. Cancer Inst., *57*: 1199–1202, 1976.
7. Gutterman, J. U., Mavligit, G., McBride, C., Frei, E., and Hersh, E. M. Immunoprophylaxis of Malignant Melanoma with Systemic BCG: Study of Strain, Dose and Schedule. Natl. Cancer Inst. Monograph *39*: 205–212, 1973.
8. Gutterman, J. U., McBride, C., Freireich, E. J., Mavligit, G., Frei, E., and Hersh, E. M. Active Immunotherapy with BCG for Recurrent Malignant Melanoma. Lancet, *1*: 1208–1212, 1973.
9. Hanna, M. G., Jr., and Peters, L. C. Efficacy of Intralesional BCG Therapy in Guinea Pigs with Disseminated Tumor. Cancer, *36*: 1298–1304, 1975.
10. Hanna, M. G., Jr., Peters, L. C., and Fidler, I. J. The Efficacy of BCG-induced Tumor Immunity in Guinea Pigs with Regional and Systemic Malignancy. Cancer Immunol. Immunotherapy, *1*: 171–177, 1976.
11. Hanna, M. G., Jr., Snodgrass, M. J., Zbar, B., and Rapp, H. Histopathology of Tumor Regression after Intralesional Injection of *Mycobacterium bovis*. IV. Development of Immunity to Tumor Cells and BCG. J. Natl. Cancer Inst., *51*: 1897–1908, 1973.
12. Hanna, M. G., Jr., Zbar, B., and Rapp, H. J. Histopathology of Tumor

Regression after Intralesional Injection of *Mycobacterium bovis*. I. Tumor Growth and Metastasis. J. Natl. Cancer Inst., *48*: 1441–1455, 1972.
13. Hawrylko, E. Immunopotentiation with BCG: Dimensions of a Specific Antitumor Response. J. Natl. Cancer Inst., *54*: 1189–1197, 1975.
14. Leibo, S. P., Farrant, J., Mazur, P., Hanna, M. G., Jr., and Smith, L. H. Effects of Freezing on Marrow-Stem Cell Suspensions: Interactions of Cooling and Warming Rates in the Presence of PVP, Sucrose and Glycerol. Cryobiology, *6*: 315–332, 1970.
15. Mazur, P., Leibo, S. P., Farrant, J., Chu, E. H. Y., Hanna, M. G., Jr., and Smith, L. H. Interactions of Cooling Rate, Warming Rate, and Protective Additive on the Survival of Frozen Mammalian Cells. *In*: G. E. W. Wolstenholme and M. O'Conner (eds.), The Frozen Cell, pp. 69–88. London: Churchill Livingston, 1970.
16. McKneally, M. F., Maver, C., and Kausel, H. W. Regional Immunotherapy of Lung Cancer with Intrapleural BCG. Lancet, *1*: 377–379, 1976.
17. Morton, D. L., Eilber, E. R., Joseph, W. L., Trahan, E., and Ketcham, A. S. Immunological Factors in Human Sarcomas and Melanomas: A Rational Basis for Immunotherapy. Ann. Surg., *172*: 740–748, 1970.
18. Pinsky, C., Hirshaut, Y., and Oettgen, H. Treatment of Malignant Melanoma by Intratumoral Injection of BCG. Natl. Cancer Inst. Monograph:, *39*: 225–228, 1973.
19. Rapp, H. J., Churchill, W. H., Jr., Kronman, B. S., Rolley, R. T., Hammond, W. G., and Borsos, T. Antigenicity of a New Diethylnitrosamine Induced Transplantable Guinea Pig Hepatoma: Pathology and Formation of Ascites Variant. J. Natl. Cancer Inst., *41*: 1–11, 1968.
20. Salmon, S. E. Immunotherapy of Cancer: Present Status of Trials in Man. Cancer Res., *37*: 1245–1248, 1977.
21. Seigler, H. F., Shingleton, W. W., Metzgar, R. S., Buckley, C. E., III, and Bergoc, T. M. Immunotherapy in Patients with Melanoma. Ann. Surg., *178*: 352–359, 1973.
22. Sokal, J. E., Aungst, C. W., and Grace, J. T., Jr. Immunotherapy of Chronic Myelocytic Leukemia. Natl. Cancer Inst. Monograph, *39*: 195–198, 1973.
23. Sokal, J. E., Aungst, C. W., and Han, T. Use of *Bacillus Calmette-Guerin* as Adjuvant in Human Cell Vaccines. Cancer Res., *32*: 1584–1589, 1972.
24. Weiner, R. S., Oldham, R. K., and Schwarzenberg, L. Cryopreservation of Normal and Neoplastic Cells, pp. 1–279. Proceedings of the International Conference. Institut National de la Santé et de la Recherche Médicale, Ministere de la Santé Publique et de la Securité Sociale, Paris, France, 1973.
25. Zbar, B., Bernstein, I. D., Bartlett, G. L., Hanna, M. G., Jr., and Rapp, H. J. Immunotherapy of Cancer: Regression of Intradermal Tumors and Prevention of Growth of Lymph Node Metastases after Intralesional Injection of Living *Mycobacterium bovis (Bacillus Calmette-Guérin)*. J. Natl. Cancer Inst., *49*: 119–130, 1972.
26. Zbar, B., and Tanaka, T. Immunotherapy of Cancer: Regression of Tumors after Intralesional Injection of *Mycobacterium bovis*. Science, *172*: 271–273, 1971.
27. Zbar, B., Wepsic, H. T., Borsos, T., and Rapp, H. J. Tumor-Graft Rejection in Syngeneic Guinea Pigs. Evidence for a Two-Step Mechanism. J. Natl. Cancer Inst., *44*: 475–481, 1970.
28. Zbar, B., Wepsic, H. T., Rapp, H. J., Borsos, T., Kronman, B. S., and Churchill, W. H., Jr. Antigenic Specificity of Hepatomas Induced in Strain-2 Guinea Pigs by Diethylnitrosamine. J. Natl. Cancer Inst., *43*: 833–841, 1969.

Exhibit E

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...ABLISHMENT OF A TUMOR-SPECIFIC IMMUNOTHERAPY MODEL UTILIZING TNP-REACTIVE HELPER T CELL ACTIVITY AND ITS APPLICATION TO THE AUTOCHTHONOUS TUMOR SYSTEM[1]

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...reinduction of potent hapten-reactive helper T ...l activity and subsequent immunization with ...pten-coupled syngeneic tumor cells result in en...nced induction of tumor-specific immunity ...ough T-T cell collaboration between anti-hapten ...lper T cells and tumor-specific effector T cells. On ...basis of this augmenting mechanism, a tumor-...ecific immunotherapy protocol was established which a growing tumor regresses by utilizing a ...tent trinitrophenyl (TNP)-helper T cell activity. ...H/He mice were allowed to generate the amplified ...ore potent) TNP-helper T cell activity by skin ...inting with trinitrochlorobenzene (TNCB) after ...treatment with cyclophosphamide. Five weeks ...er, the mice were inoculated intradermally with ...ng... ...c transplantable X5563 tumor cells. When ... ...s injected into X5563 tumor mass, an ap... ...ble number of growing tumors, in the only ...up of C3H/He mice in which the amplified TNP-...lper T cell activity had been generated were ob...rved to regress (regressor mice). These regressor ...ce were shown to have acquired tumor-specific T ...ll-mediated immunity. Such immunity was more ...tent than that acquired in mice whose tumor was ...nply removed by surgical resection. These results ...licate that *in situ* TNP haptenation of the tumor ...lls in TNP-primed mice can induce the enchanced ...mor-specific immunity leading to the regression ...a growing tumor. Most importantly, the present ...udy further investigates the applicability of this ...NP immunotherapy protocol to an autochthonous ...mor system. The results demonstrate that an ap...eciable percent of growing methylcholanthrene-...duced autochthonous tumors regressed by the ...ove TNP immunotherapy protocol. Thus, the pres...t model provides an effective maneuver for tumor-...ecific immunotherapy in syngeneic transplanta...n as well as autochthonous tumor systems.

On the basis of the hypothesis of Mitchison (1) con...rning manipulations that might augment tumor-spe...ic immunity, numerous attempts to enchance the im-munogenicity of tumor-associated transplantation antigens (TATA)[3] by coupling additional antigenic determinants on the tumor cell surface have been reported (2–6). Helper T cells can collaborate with effector T cell precursors, such as cytotoxic cell precursors, to enhance immune responses against various antigens including TATA (7). If additional determinants coupled onto the tumor cell act as helper determinants, it is therefore conceivable that preinduction of helper T cell activity to these additional determinants could induce much higher anti-TATA immune responses at the time of stimulation of tumor cells conjugated with the corresponding antigenic determinants.

We defined conditions under which enhanced immune resistance to tumors could be generated by preinducing trinitrophenyl (TNP) hapten-reactive T cells, and by subsequently immunizing with TNP-coupled syngeneic tumor cells (8, 9). This system is designed to induce the most efficient generation of tumor-specific effector T cell activity *in vivo* by virtue of the close linkage of hapten-reactive helper T cells and TATA-specific effector precursor T cells in the microenvironment at the time of stimulation with hapten-coupled tumor cells. Our previous results demonstrated that the generation of potent TNP-helper cell activity after elimination of suppressor cell activity was a prerequisite for amplified generation of *in vivo* protective immunity, and a T-T cell interaction mechanism between TNP-helper T cells and anti-TATA effector T cell precursors was thus suggested to be essential to such a phenomenon (10). These results prompted us to establish an immunotherapeutic protocol in tumor-bearing animals in which such potent TNP-helper T cells were used.

In the present study, when TNP was introduced into the tumor mass of tumor-bearing mice in which the amplified TNP-reactive helper T cell activity had been generated, *in situ* trinitrophenylation of tumor cells resulted in a high incidence of complete regression of growing tumors. We demonstrated that the tumor regression was accompanied by the concurrent generation of a potent tumor-specific T cell immunity, suggesting on the above T-T cell collaboration mechanism was functioning in this tumor immunotherapy protocol. More importantly, the present study also investigates whether such an immunotherapeutic potential realized in the TNP-helper

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[3] Abbreviations used in this paper: TATA, tumor-associated transplantation antigens; TNCB, trinitrochlorobenzene; MCA, 3-methylcholanthrene; Cy, cyclophosphamide; CTL, cytotoxic T lymphocyte; i.d., intradermal; DTH, delayed-type hypersensitivity.

TABLE I

Comparison of acquisition of tumor-specific immunity after TNP immunotherapy and surgical resection of tumor

Group	Mice	Incidence of Resistance against Tumor Challenge[a]
A	Normal	0/10
B	After regression of tumor by TNP immunotherapy[b]	11/12
C	After surgical resection of tumor[c]	2/10

[a] Mice were challenged i.d. with 10^6 viable X5563 tumor cells, and incidence of resistance was determined 3 weeks after the tumor challenge.

[b] C3H/He mice whose X5563 tumor regressed in the TNP immunotherapy model as shown in Fig. 1 (group E) were used 3 wk after initial tumor implantation.

[c] C3H/He mice were inoculated i.d. with 10^6 X5563 tumor cells and growing tumors were surgically resected 7 days later. Mice were used 2 wk after tumor removal.

growth of metastasized tumor cells. Thus, the difference in incidence of anti-X5563 immunity between two groups above indicates more potent anti-X5563 immune resistance was retained in mice whose tumors regressed by virtue of the TNP immunotherapy.

The development of stronger anti-X5563 immune resistance in regressor mice was also confirmed by comparing the tumor-neutralizing activity of spleen cells from these mice to that of mice whose tumors were surgically resected. Winn assay performed with these two groups of spleen cells at a lower spleen to tumor cell ratio [illegible] that appreciably stronger tumor-neutralizing activity was generated in the regressor mice by TNP immunotherapy than in the mice that had tumors resected surgically (Table II).

Additional experiments were performed to test the nature and specificity of the effector mechanism acquired by X5563 tumor regressor mice. Winn assays with the use of spleen cells from the regressor mice also demonstrated that these spleen cells resulted in complete neutralization of X5563 tumor cells when admixed, but failed to exhibit 1) tumor neutralization against X5563 tumor after the treatment of the spleen cells with anti-Thy-1.2 plus C (Table III), and 2) tumor neutralization against another syngeneic tumor MH134 hepatoma (Table IV). These results indicate the T cell nature and specificity of anti-X5563 immunity acquired by the regressor mice in the TNP immunotherapy model.

Application of the TNP immunotherapeutic protocol to an autochthonous tumor system. In the process of application of the present tumor-specific immunotherapy model to a chemical carcinogen-induced autochthonous tumor system, we extended this TNP immunotherapy model to another transplantable, chemical carcinogen-induced tumor (MCH-1-A1) system in which the tumor was recently induced in C3H/He mice by MCA and has been maintained in our laboratory (less than 10 passages *in vivo*). A similar protocol to that performed in the X5563 tumor system was used and the results are illustrated in Figure 2. In this experiment, TNCB injection into the MCH-1-A1 tumor mass from Cy→TNCB-painted mice led to a high incidence of tumor regression, in contrast to the lack of tumor regression when *in situ* TNP modification was performed in mice not primed to TNP. Thus, this TNP immunotherapy system is also applicable to another recently established transplantable, chemical carcinogen-induced fibrosarcoma tumor system.

The successful regression of growing tumors in an MCA-induced transplantable tumor system by using the TNP immunotherapy regimen encouraged us to test the applicability of this immunotherapy protocol to an MCA-induced autochthonous tumor system. The primary tumor was induced in 500 female C3H/HeN mice at 8 wk of age by injecting 0.5 mg MCA in 0.1 ml olive oil subcutaneously. Four weeks after the MCA inoculation, one half of the group of mice received the combined treatment of Cy injection and TNCB painting, which was capable of inducing the amplified TNP-reactive helper T cell activity, and the remainder were untreated. The mice began to develop a primary, subcutaneous tumor about 8 wk after the MCA treatment. At 9 wk after the MCA injection, 20 to 30% of mice in both TNP-helper-positive and -negative groups bore a tumor in the range of 6 to 9 mm in diameter. Histological examination of 10 autochthonous tumors randomly selected (five mice in each group) revealed that all were fibrosarcoma. Mice that did not receive tumor excision were collected and each group was randomly divided into two groups depending on whether mice were treated with the intratumoral injection of 0.15 ml of 1% TNCB. Therefore, the experiment consisted of four groups: group A: MCA injection only; group B: MCA injection→intratumoral TNCB injection; group C: MCA injection→the combined treatment of Cy plus TNCB painting; and group D: MCA injection→the above combined treatment for priming of potent TNP-helper T cells→intratumoral TNCB injection. The tumor growth of four groups of animals is shown in Figure 3. Most tumors in three groups of mice (groups A, B and C), except for only one animal in group B, continued to grow until the animal died, although the growth rate exhibited varied patterns. Importantly, however, an appreciable number (11 of 25)

TABLE II

Comparison of tumor-neutralizing activity between spleen cells from mice after tumor regression after TNP immunotherapy and from mice after surgical resection of tumor

Spleen Cells from Mice	Spleen:Tumor Cell Ratio	Tumor Growth[a] (mm diam)		
		Day 7	Day 10	Day 12
[illegible]tion of tumor by TNP immunotherapy[b]	100:1	5.8 ± 1.3	9.6 ± 1.0	13.5 ± 0.3
[illegible]al resection of tumor[c]	100:1	<3.0	<3.0	<3.0
	100:1	<3.0	<3.0	<3.0
After regression of tumor by TNP immunotherapy	10:1	4.8 ± 0.9	9.3 ± 0.9	12.5 ± 1.0
After surgical resection of tumor	10:1	<3.0	<3.0	5.0 ± 1.5
	10:1	<3.0	7.5 ± 0.5	10.3 ± 1.2

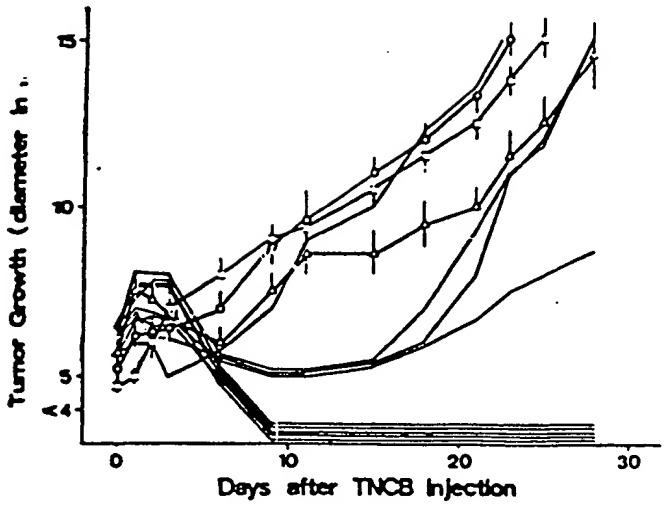

Figure 2. Induction of tumor regression in MCH-1-A1 tumor-bearing mice by using the TNP immunotherapy regimen. C3H/He mice received the combined treatment of Cy injection and TNCB painting. Five weeks after TNCB painting, mice were inoculated i.d. with 10^6 viable MCH-1-A1 tumor cells. The *in situ* TNP haptenation identical to that in Fig. 1 was performed 7 days after tumor cell inoculation. Tumor growth was individually scored and expressed by tumor diameter (——). Tumor growth in control groups was expressed by mean diameter ± SE of seven mice per group. (O——O), (Δ——Δ), and (□——□) indicate tumor cell inoculation only, tumor cell inoculation→*in situ* TNP haptenation, and the above combined treatment for TNP priming→tumor cell inoculation, respectively.

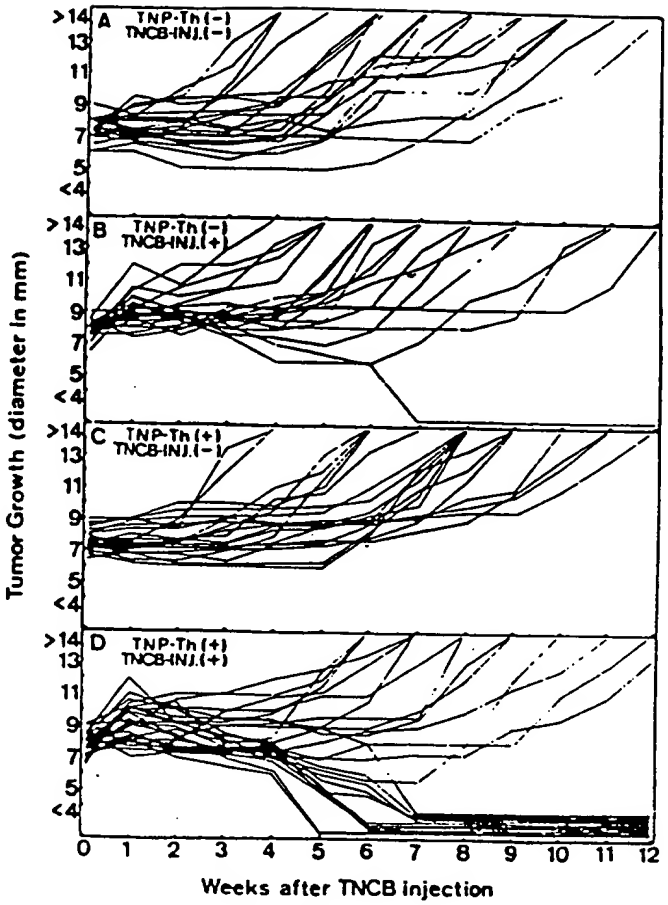

Figure 3. Regression of growing autochthonous tumors by the TNP immunotherapy regimen. C3H/He mice were inoculated subcutaneously with 0.5 mg MCA. Four weeks later, mice received the combined treatment of Cy injection and TNCB painting (groups C and D). Nine weeks after MCA injection, 0.15 ml TNCB in olive oil was administered into subcutaneously growing autochthonous tumors of groups B and D. Group A was MCA inoculation only. Tumor growth was individually scored and expressed by tumor diameter. Tumors in all groups that reached a 14-mm diameter continued to grow for as many as 8 wk ultimately killing the animal. For limitation of the scale, such stage of growth was omitted.

TABLE V

Summary of incidence of tumor regression and mean survival time[a]

Group	Treatment		Incidence of Tumor Regression	Mean Survival Time (weeks ± SE)	No. Dead Mice
	TNP-Th Induction	TNCB Injection			
A	−	−	0/20	13.00 ± 0.75	20
B	−	+	1/20	11.32 ± 0.55	19
C	+	−	0/20	13.60 ± 0.58	20
D	+	+	11/25	12.90 ± 0.68	14

[a] Determined 20 wk after injection of TNCB into autochthonous tumor and expressed by mean survival time of dead mice at this stage.

T cell immunity was more potent in the tumor-regressed than in mice whose tumor was surgically resected.

ould also be noted that X5563 tumor-specific im-unity, which had been acquired in tumor-regressed mice, was mediated by anti-X5563-TATA-specific Lyt-1^+2^-, but not by Lyt-1^-2^+ T cells, indicating that the tumor-specific Lyt-1^+2^- T cell population whose generation was augmented through collaboration with TNP-specific helpers primarily exhibited a protective effect (T. Yoshioka, H. Fujiwara, and T. Hamaoka, manuscript in preparation). Because these Lyt-1^+2^- T cells exhibited no cytotoxic effect on X5563 tumor cells in a 4-hr ^{51}Cr-release assay, further studies are in progress concerning the mechanisms of anti-tumor-specific Lyt-1^+ T cell function in eradicating tumor cells *in vivo*.

The most interesting and important finding in the present study [which has not been reported in other tumor-specific immunotherapy experiments] is the demonstration of the applicability of this TNP immunotherapy protocol to an autochthonous tumor system. This finding is worthy of discussion from two perspectives. First, the evidence that TNP immunotherapeutic potential allows the induction of tumor regression to an appreciable proportion in autochthonous as well as transplantable tumor systems clearly emphasizes the validity of this TNP immunotherapy model on the basis of the T-T cell interaction mechanism. This could also provide a theoretical basis for Klein's clinical approaches in which skin malignancies were treated by haptenic reagents (16). Although further investigation is required to explore a chemical t suitable for the *in situ* modification of human ...rs, the present system may provide a prototye of the immunotherapy of some types of clinical tumors such as skin cancers.

Second, it remains to be proven why 14 of 25 of the autochthon... been assumed that most of tumors bear TATA (17, 18), the qualitative diversity and quantitative heterogeneity in the expression of each putative TATA on an autochthonous tumor cell has not been well determined. Further experiments are therefore required to determine whether the tumor-specific immunity is in fact acquired in mice whose autochthonous tumor has regressed and how putative TATA in each individual autochthonous tumor qualitatively varies, and to investigate the relationship between the immunogenicity of the autochthonous tumor and the prognosis of the tumor-specific immunotherapy. Such approaches are in progress by challenging the autochthonous tumor cells obtained by excisional biopsy

Exhibit F

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COMMUNICATION

The Induction of Cytolytic T Lymphocytes with Syngeneic Trinitrophenyl-Coupled Membranes[1]

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Recently we have demonstrated the induction of allogeneic murine cytolytic T lymphocytes (CTL)[3] using purified plasma membranes rather than intact cells as stimulatory agents (1). In this report we extend the use of such subcellular preparations to study the requirements for hapten-specific syngeneic CTL induction. Membranes prepared from trinitrophenyl (TNP) coupled syngeneic tumor cells retain the ability to stimulate both a primary and secondary CTL response. The CTL that are generated are restricted in their lysis to target cells bearing the same H-2 antigens as those present on the TNP-coupled stimulating membranes.

MATERIALS AND METHODS

All materials and methods used in the *in vitro* induction and assay of TNP specific CTL are as previously described (2). Briefly, 7×10^6 spleen cells from nonimmune or immune mice were co-cultured with x-irradiated, TNP-coupled spleen cells or TNP-coupled membranes. After 5 days of culture cells were harvested and cytolytic activity was assessed in a 4-hr assay against 10^4 ^{51}Cr-labeled TNP-coupled tumor targets or LPS blast cell targets. Immune spleen cells were obtained by priming mice subcutaneously with 2×10^7 TNP-coupled autologous spleen cells 2 weeks before *in vitro* culture. Membranes used in stimulation of CTL were prepared from TNP coupled DBA/2 mastocytoma P815 (H-2^d) tumor cells or from TNP-coupled C57BL/6 (B6) leukemia EL-4 (H-2^b) tumor cells. Purified plasma membranes were used for CTL induction in the experiment described in Table I. The results presented in Tables II and III were obtained by using partially purified plasma membranes referred to as "high speed pellet" in Reference 1. Spontaneous ^{51}Cr release ranged from 30 to 39% for LPS-induced blast cell targets and from 11 to 19% for tumor cell targets.

RESULTS

Plasma membranes prepared from TNP-modified tumor cells were tested for their ability to stimulate both primary and secondary CTL responses by using H-2 syngeneic responder cells. As demonstrated in Tables I and II, such membranes were active in the stimulation of primary and secondary hapten specific CTL. CTL generated by coupled membranes are similar in specificity to those generated by coupled cells in that they preferentially lyse syngeneic target cells (2–4). These membranes stimulated variable amounts of cross-reactive lysis on TNP coupled allogeneic target cells. B6 spleen cells stimulated with TNP-EL-4 membranes did not lyse targets that did not bear TNP. Lytic activity was not induced when B6 spleen cells were co-cultured with uncoupled EL-4 membranes.

It has been recently demonstrated (5, 6) that cells incubated with TNP-coupled proteins are capable of stimulating a hapten-specific cytolytic response that is restricted to target cells that are H-2 identical with the responder cell population. Therefore, it was important to determine if stimulation by the membranes was dependent on the H-2 antigen present on the membrane, or whether the membrane proteins were simply contributing the hapten that was then recognized in conjunction with the H-2 antigens of the responder cell population. B6D2F$_1$ (H-$2^{b/d}$) immune spleen cells were stimulated with either TNP-EL-4 membranes or TNP-P815 membranes and the specificity of the resultant CTL was studied. It would be expected that if the H-2 present on the membranes did not influence the specificity of the CTL, then in either case the CTL would lyse both B6-TNP (H-2^b) and B10.D2-TNP (H-2^d) targets to a similar extent. As is shown in Table III the CTL preferentially lyse target cells that bear the same H-2 antigens as the TNP-membranes used in CTL stimulation. Similar specificity was obtained with CTL resulting from stimulation of nonimmune B6D2F$_1$ spleen cells (Table II). These results indicate that both the TNP and the H-2 antigens present on the membranes determine the specificity of the CTL population.

DISCUSSION

The results described above extend the use of subcellular material to the study of CTL recognition in a chemically modified syngeneic system. The results demonstrate the capacity of membranes prepared from TNP-modified tumor cells to induce primary and secondary CTL having the same specificity as CTL that are induced by TNP-coupled cells. The ability to stimulate B6D2F$_1$ CTL that are restricted in their recognition to the H-2 antigens present on the stimulating membrane

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[3] Abbreviations used in this paper: CTL, cytolytic T lymphocytes; H-2, histocompatibility complex-2; B6D2F$_1$, (C57BL/6 × DBA/2)F$_1$; B6, C57BL/6.

TABLE I

Specificity of secondary (BALB/c × DBA/2)F₁ (H-2ᵈ) CTL stimulated by TNP-coupled membranes

Stimulator[a]	% Specific ^{51}Cr Release			
	P815-TNP (H-2ᵈ)		EL-4-TNP (H-2ᵇ)	
	25/1[b]	12.5/1	25/1	12.5/1
Experiment 1				
—	19	9		
12-μg membranes	32	19		
24-μg membranes	44	24		
72-μg membranes	59	38		
Experiment 2				
—	31	19	19	12
BALB/c-TNP cells	83	61	40	14
3-μg membranes	31	16	13	8
10-μg membranes	41	19	17	11
30-μg membranes	63	40	22	14

[a] Membranes used for this experiment were purified plasma membranes obtained from TNP-coupled P815 tumor cells (1).

[b] Effector to target ratio.

TABLE II

Induction of primary (C57BL/6 × DBA/2)F₁ (H-2ᵇ/ᵈ) CTL by TNP-coupled membranes

Stimulator[a]	% Specific ^{51}Cr Release[b]	
	B6-TNP (H-2ᵇ)	B10.D2-TNP (H-2ᵈ)
—	7	7
B6-TNP cells	64	34
24-μg membranes	28	12
75-μg membranes	33	6
150-μg membranes	38	6

[a] Membranes used in this experiment were partially purified from TNP-coupled EL-4 tumor cells (H-2ᵇ).

[b] Effector to target ratio is 50:1. Target cells were LPS-stimulated blast cells.

TABLE III

Specificity of TNP-membrane-induced CTL

Responder	Stimulator	% Specific ^{51}Cr Release Targets[a]	
		B6-TNP (H-2ᵇ)	B10.D2-TNP (H-2ᵈ)
Primed B6D2F₁ (H-2ᵇ/ᵈ)	—	6	9
	75 μg EL-4-TNP membranes	29	10
	84 μg P815-TNP membranes	16	32

[a] Effector to target ratio was 50:1. Targets were LPS-stimulated blast cells.

preparations indicates that induction results from recognition of both the H-2 and the hapten on the membranes and not from haptenated protein(s) from the membranes that associate with the responder cells. In this regard, TNP membranes are similar in their inductive capacity to TNP cells. It has been previously reported that H-2 antigens need not be directly haptenated in order to obtain a CTL response. Recent experiments that have utilized TNP-coupled serum proteins to stimulate TNP-specific CTL have argued against the contention by Forman *et al.* (7) that only TNP present on H-2 is antigenically active. Although the experiments described above do not address the question of a requirement for direct haptenization of H-2 to stimulate, it is clear that TNP-membranes are antigenically similar to TNP-cells rather than TNP-proteins.

It is also of interest to consider these results as they address the mechanism of CTL stimulation by subcellular material. The possibility exists that stimulation of CTL by subcellular preparations occurs via presentation of antigen by intact cells present in the cultures (e.g., macrophages) rather than by direct interaction between the membrane vesicle and pre-CTL. It is clear that if indeed material must be presented by viable cells to be antigenic, these cells do not determine the specificity of the resulting CTL.

Ozata and Henney (6) have reported that membranes from TNP-coupled spleen cells failed to induce a secondary syngeneic CTL response whereas the results shown in Tables I and II of this report clearly show that membranes from TNP-coupled tumor cells can induce a specific secondary response. This discrepancy might be accounted for by the difference in cell type used as a membrane source.

The ability to stimulate CTL with TNP-modified membranes opens the possibility that we will be able to isolate, in a soluble form, TNP-modified membrane proteins that retain biologic activity (i.e., the ability to induce CTL) (8). One could then determine whether an effective immunogen is created by TNP-modified non-MHC proteins that interact with H-2, or whether direct chemical modification of H-2 antigens creates the immunogen, or whether both possibilities exist.

SUMMARY

Evidence is presented that trinitrophenyl-coupled tumor membranes are able to induce cytolytic T lymphocytes (CTL) when co-cultured with syngeneic spleen cells. These haptenated membranes stimulate spleen cells from naive and immune mice. The specificity of these CTL is determined by the H-2 antigens of the membranes used for stimulation.

REFERENCES

1. Lemonnier, F., M. Mescher, L. Sherman, and S. Burakoff. 1978. The induction of cytolytic T lymphocytes with purified plasma membranes. J. Immunol. 120:1114.
2. Burakoff, S. J., R. N. Germain, and B. Benacerraf. 1976. Cross-reactive lysis of trinitrophenyl (TNP)-derivatized H-2 lymphocytes generated against syngeneic TNP spleen cells. J. Exp. Med. 144: 1609.
3. Shearer, G. N., T. G. Rehn, and A. M. Schmitt-Verhulst. 1976. Role of murine major histocompatibility complex in the specificity of *in vitro* T cell-mediated lympholysis against syngeneic trinitrophenyl-modified targets. Transplant. Rev. 29:222.
4. Forman, J. 1975. On the role of the H-2 histocompatibility complex in determining the specificity of cytotoxic effector cells sensitized against syngeneic trinitrophenyl-modified targets. J. Exp. Med. 142: 403.
5. Schmitt-Verhulst, A. M., C. B. Pettinelli, P. A. Henkart, J. K. Lunney, and G. M. Shearer. 1978. H-2 restricted cytotoxic effectors generated *in vitro* by the addition of trinitrophenyl-conjugated soluble proteins. J. Exp. Med. 147:352.
6. Ozato, K., and C. S. Henney. 1978. Studies on lymphocyte-mediated cytolysis. XII. Hapten transferred to cell surfaces by interaction with liposomes is recognized by antibody but not by hapten-specific H-2 restricted cytotoxic T cells. J. Immunol. 121:2405.
7. Forman, J., E. S. Vitetta, and D. A. Hart. 1977. Relationship between trinitrophenyl and H-2 antigens on trinitrophenyl-modified spleen cells. J. Immunol. 118:803.
8. Mescher, M., L. Sherman, F. Lemonnier, and S. Burakoff. 1978. The induction of secondary cytolytic T lymphocytes by solubilized membrane proteins. J. Exp. Med. 147:946.

Exhibit 17

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Adjuvant Active Specific Immunotherapy for Human Colorectal Cancer: 6.5-Year Median Follow-Up of a Phase III Prospectively Randomized Trial

By Herbert C. Hoover, Jr, Jane S. Brandhorst, Leona C. Peters, Mildred G. Surdyke, Yoshiko Takeshita, Juan Madariaga, Larry R. Muenz, and Michael G. Hanna, Jr

Purpose: Patients with colon or rectal cancer were entered onto a prospectively randomized, controlled clinical trial of active specific immunotherapy (ASI) with an autologous tumor cell–bacillus Calmette-Guérin (BCG) vaccine. We investigated whether ASI could improve disease-free status and survival.

Patients and Methods: Ninety-eight patients with Dukes' stage B_2-C_3 colon or rectal cancer were randomized into groups treated by resection alone or resection plus ASI. Eighty patients met all eligibility criteria. All patients with rectal cancer were to receive 50 Gy of pelvic irradiation. Analysis of distribution of survival and disease-free survival was made on all eligible patients until December 31, 1990.

Results: As a single study, no statistically significant differences were detected in survival or disease-free survival for all 80 eligible patients. However, since it was recognized at the outset that there were treatment differences, in that rectal cancer patients were to receive postimmunotherapy radiation, it was considered that a cohort analysis of the colon and rectal cancer patients might be informative. With a median follow-up of 93 months, there is a significant improvement in survival (two-sided $P = .02$; hazards ratio, 3.97) and disease-free survival (two-sided $P = .039$; hazards ratio, 2.67) in all eligible colon cancer patients who received ASI. With a median follow-up of 58 months, no benefits were seen in patients with rectal cancer who received ASI.

Conclusion: This study suggests that ASI may be beneficial to patients with colon cancer.

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COLORECTAL CANCER continues to be one of the most prevalent malignancies in the United States, with more than 157,000 new cases diagnosed and more than 60,000 deaths occurring each year.[1] Although surgical resection is usually the only curative therapy, established gross metastases and micrometastases commonly frustrate these efforts. Despite improved surgical approaches, patients with transmural extension of tumor and metastases to regional lymph nodes still have 5-year survival rates of only 30% to 50% when treated by operative resection alone. Adjuvant radiation therapy can lower the incidence of local recurrence, but does not control systemic metastasis, the cause of death in many of these patients.[2-4]

Some recently published trials indicate benefit from chemotherapy, especially a combination of fluorouracil (5-FU) and levamisole in Dukes' stage C colon carcinoma,[5,6] and a combination of radiation therapy and 5-FU/methyl-lomustine (CCNU) chemotherapy for rectal carcinoma.[7] However, systemic toxicity and drug resistance continue to limit the usefulness of chemotherapy. New approaches are needed to engender further advances.

Biologic therapy or biologic response modifier therapy has moved beyond the era of nonspecific immunotherapy that laid some of the foundation for today's approaches. Our efforts have focused on an approach that makes use of a patient's own tumor to elicit an immune response. This approach is known as active specific immunotherapy (ASI). There is now extensive evidence that many human tumors have tumor-associated antigens, although assessment of specificity of these antigens remains difficult.[8] In our use of ASI, we are presuming the presence of distinct tumor antigens on tumor cells that are absent on normal cells. We are attempting to activate the host defenses against these antigenic factors, which are theoretically distinctive to each tumor, by increasing the immunogenicity of autologous tumor cells with an immunomodulating adjuvant, bacillus Calmette-Guérin (BCG).

The impetus for this work was the development and biologic characterization of an experimental model that determined the requirements for effective immunotherapy of established tumor. A series of studies in a guinea pig line-10 hepatocarcinoma model[9-15] demonstrated that BCG admixed with syngeneic tumor cells can induce systemic immunity capable of eliminating a limited disseminated tumor burden when the vaccine is carefully controlled for variables such as the number of tumor cells.

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ratio of viable BCG organisms to tumor cells, viability of the tumor cells, and vaccination regimen.

A five-patient pilot study in 1980 demonstrated that we could successfully dissociate human colorectal cancers into viable cell preparations and that the toxicity of the tumor cell-BCG vaccine was minimal. The principles and procedures of ASI, as learned in the guinea pig hepatocarcinoma model, were then applied in a randomized, controlled trial of patients with colorectal cancer. The decision was made to start this trial in a prospectively randomized, controlled manner to maximize the information achieved from an extraordinarily labor-intensive and expensive trial. The study began at the Johns Hopkins Hospital, Baltimore, MD, in 1981, was transferred to University Hospital, Stony Brook, NY, in 1983, and then to the Massachusetts General Hospital, Boston, MA, in 1986. Accrual ended in November 1988 and follow-up extended to December 1990. The objectives of this study were to determine whether ASI could (1) enhance the delayed cutaneous hypersensitivity (DCH) response to autologous tumor cells, and (2) prolong the disease-free survival of these patients. We have reported[16] that immunized patients showed a significant increase in the DCH response to autologous tumor cells compared with autologous normal mucosa, used as a normal tissue control. Furthermore, no significant DCH responses against autologous tumor or mucosa cells were detected in a control group of nonimmunized patients. The qualitative and quantitative differences in DCH responses to tumor cells and to normal mucosa cells suggest that the immunizations are targeted mainly to tumor-associated antigens, and that tissue-associated antigens play a secondary role.

Correction to the Original Report[17]

In 1985,[17] we reported the preliminary clinical results with a mean follow-up of 28 months. In preparing the follow-up report on that work, we were made aware of oversights in the 1985 report that we now wish to correct. In 1985, we failed to state that there were patients randomized onto the trial who were later excluded as being ineligible. These patients are now listed to allow the reader to account for every patient randomized. In this final audit, eligibility criteria have been more rigidly defined, requiring source documentation for all reported data. Thus, some patients included in the original report have been reclassified as to their eligibility. These changes have resulted in a total of 42 eligible patients at the time of the 1985 interim analysis, rather than the 40 patients reported. The net change of two patients results from the reclassification of six patients. The reclassified patients included two control patients who were later found to be ineligible and four treated patients who were reinstated.

The trial was established as simultaneous studies for colon and rectal cancer patients. In the 1985 analysis, all patients were combined for analysis because of the small number of patients. With the reclassification and a more appropriate data analysis, the major conclusion of the 1985 report is altered in that the apparent advantage of immunotherapy is limited to patients with colon cancer. There is no perceived benefit in rectal cancer patients. On July 31, 1984, of 27 colon cancer patients, 38% of control and 14% of treated patients had recurred and 23% and 0%, respectively, had died. Of 15 rectal cancer patients, 60% of control and 50% of treated patients had recurred and 0% and 10%, respectively, had died. Statistical analysis of such small numbers has little power to detect differences, but the encouraging advantage in the colon cancer patients who received immunotherapy is suggested.

This report updates that clinical trial with a total of 80 eligible patients. It accounts for every patient randomized and elaborates on the methodology used for the entire trial.

PATIENTS AND METHODS

Patient Selection and Randomization

Eligible patients were those with colon or rectal cancers extending through the bowel wall (Gunderson and Sosin[18] stages B_2 or B_3) or with positive lymph nodes (stages C_1, C_2, or C_3) (Table 1) for whom adequate cells from the primary tumor were available. Rectal cancer was defined as any tumor involving or below the pelvic peritoneal reflection. Suitable medical condition to comply with the outpatient treatment protocol was required (Eastern Cooperative Oncology Group [ECOG] performance status 0 or 1). From 1981 until 1983, patients with a history of cancers controlled by surgery, chemotherapy, or radiation therapy greater than 5 years from entry into the study were considered eligible. Since 1983, to avoid any chance of confusing a recurrence from a prior cancer, patients with any prior cancer (except basal or squamous cell skin cancer) have been excluded. The issue of prior malignancies is the only change in the eligibility criteria as the study progressed. Resection of the tumors was performed at Johns Hopkins Hospital, Loch Raven Veterans Hospital, Baltimore City Hospital, St Agnes Hospital, and Greater Baltimore Medical Center, all in Baltimore, MD; University Hospital, Stony Brook, NY; Peninsula General Hospital, Far Rockaway, NY; Northwestern Memorial Hospital, Chicago, IL; and Massachusetts General Hospital, Boston, MA. These studies were approved by each hospital's human studies institutional review board before any patient was entered. Informed consent was obtained from each subject. Although patients were accrued from nine hospitals, 82% of the vaccine preparations were made, and ASI patients treated, under the direct supervision of the principal investigator (H.C.H.) at Johns Hopkins, Stony Brook, and Massachusetts General. The remaining 18% were performed at Northwestern and Peninsula General, by personnel trained and quality-controlled by the principal investigator's staff, thus minimizing

site-to-site variability in vaccine preparation and administration. Figure 1 shows the schema for stratification and randomization. Randomization was centrally controlled at the institution of the principal investigator from a single set of sealed envelopes. Colon cancer patients were randomized separately from rectal cancer patients with substratification within each of the pathologic stages B_2 through C_3. Randomization cards were sequentially drawn in a blinded fashion by a protocol nurse from each category at the time (postoperatively) the patient was determined to be eligible and signed an informed consent form. An initial target sample size was 100 patients.

Surgical and Pathology Quality Control

Operative and pathology reports were reviewed carefully for adequacy of the surgical procedure and accuracy of staging, prompting a direct communication with the surgeon or pathologist in any case where questions developed. Each eligible patient had wide removal of the involved bowel segment, as well as the lymphatic drainage area. Involved adjacent organs were resected en bloc. All patients had histologically proven clear margins. Five patients were equivocal between B_1 and B_2 stages and have been called B_2 for this protocol.

Preparation of Autologous Vaccines From Solid Primary Tumors

After resection, the bowel specimen was taken immediately to the hospital pathology department and opened under sterile conditions. All tumor tissue not required for staging and 2 to 3 g of distal normal colon mucosa were excised, placed in sterile tubes with Hanks' balanced salt solution containing 50 μg gentamicin/mL, and carried immediately on ice to the laboratory for dissociation and freezing using the method reported by Peters et al.[14] The importance of minimizing damage to the metabolic activity of the cell by careful and timely dissociation and optimal cryopreservation has been addressed previously.[14-17]

A sample of the enzyme-dissociated cell preparation was sent to bacteriology for routine culture as previously described[16] or was cryopreserved along with the vaccine and later thawed for bacteriologic testing.

Clinical Protocol

Patients with tumors of the appropriate pathologic stages were randomized postoperatively either to receive the autologous tumor cell-BCG vaccine or to receive no vaccine (Fig 1). Colon cancer and rectal cancer patients were in separate but parallel studies that were identical except that postoperative pelvic irradiation was recommended for all patients with rectal cancer. Before randomization, we attempted to screen all consenting patients by chest x-rays, liver ultrasound or computerized axial tomography scans, and carcinoembryonic antigen (CEA) tests to identify patients with unsuspected metastatic disease. The vaccines were started at 4 to 5 weeks after tumor resection to allow the patient sufficient time to recover from immunologic suppression, which may be induced by anesthesia and surgery. At 3 to 4 weeks after resection, both control and immunized patients were skin-tested with standard recall antigens to evaluate immunocompetence and sensitization to tuberculin purified protein derivative (PPD). When sufficient cells were available, patients were also skin-tested with graded doses (10^4, 10^5, and 10^6) of autologous tumor and mucosa cells. After 1984, only the 10^6 dose was used, as the lower doses proved uninformative. Repeat skin testing with tumor cells and mucosa was performed at 6 weeks, 6 months, and 1 year following vaccination when sufficient autologous material was available as described previously.[16] Repeat recall antigen testing was performed at 6 weeks when possible.

Treated patients received one intradermal vaccination per week for 2 weeks consisting of 10^7 viable, irradiated, autologous tumor cells and 10^7 viable BCG organisms, as determined by colony-forming units. (Fresh-frozen TICE BCG was supplied by [illegible]on Teknik-

Table 1. Characteristics of the ASI Treatment Groups (eligible patients only)

Characteristic	Immunotherapy (n = 41) n	%	Control (n = 39) n	%
Age, years				
Mean	63		61	
Range	31-87		36-80	
Sex				
Male	27	66	24	62
Female	14	34	15	38
Race				
White	35	85	31	79
Black	5	12	5	13
Other	1	2	3	8
Location of primary*				
Right colon	10	24	11	28
Left colon	14	34	12	31
Rectal	17	41	16	41
Astler-Coller stage				
Colon				
B_2 (transmural)	14	34	11	28
B_3 (adjacent organ)	1	2	2	5
C_1 (+node, not transmural)	2	5	0	0
C_2 (B2 with +node)	6	15	8	20
C_3 (B3 with +node)	1	2	2	5
Rectal				
B_2 (transmural)	4	10	6	15
B_3 (adjacent organ)	0	0	0	0
C_1 (+node, not transmural)	4	10	2	5
C_2 (B2 with +node)	9	22	8	20
C_3 (B3 with +node)	0	0	0	0
Size of primary, cm				
Mean	5.2		5.3	
Range	3.0-10.0		3.0-11.5	
Proximal margin, cm				
Mean	15.4		16.3	
Range	2.4-40		[illegible]	
Distal margin, cm				
Mean	8.6		6.8	
Range	1.4-25		0.5-18.5	
No. of +nodes (stage C only)				
Mean	3.2		3.7	
Range	1-13		1-23	

Abbreviation: +node, positive node.

*Right colon includes bowel from cecum to midtransverse colon, left colon from midtransverse to peritoneal reflection, rectum below peritoneal reflection to anus.

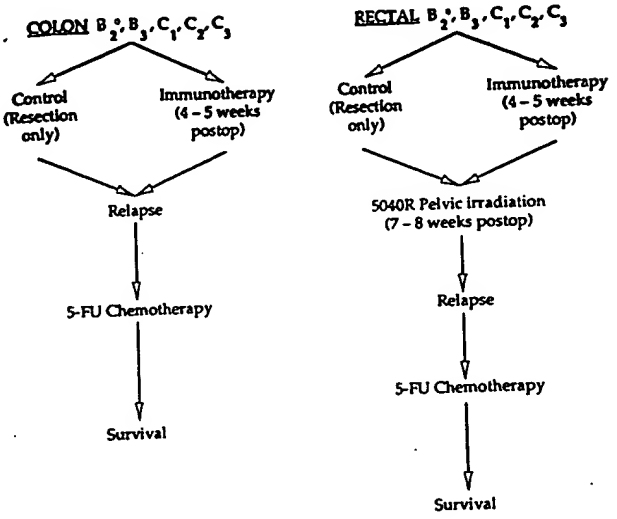

Fig 1. Schema for postoperative adjuvant ASI for colorectal cancer. Patients were grouped according to the tumor site (colon or rectal). *Each pathologic stage group was then randomized separately.

Corporation, Chicago, IL, and was stored at − 70°C.) In the third week, patients received one vaccination of 10^7 irradiated tumor cells alone. The first vaccine was placed on the left anterior thigh approximately 10 cm below the groin crease, the second in a comparable location on the right thigh, and the third in the right deltoid area. The upper thigh site was chosen because of the large lymph node basin in the immediate drainage area.

On the days of vaccination, the frozen tumor cells were thawed and the vaccines prepared according to procedures detailed previously.[15-17] The tumor cells were irradiated with 200 Gy before administration. Experience in the guinea pig model has shown that irradiation destroys the tumorogenicity, but not the immunogenicity

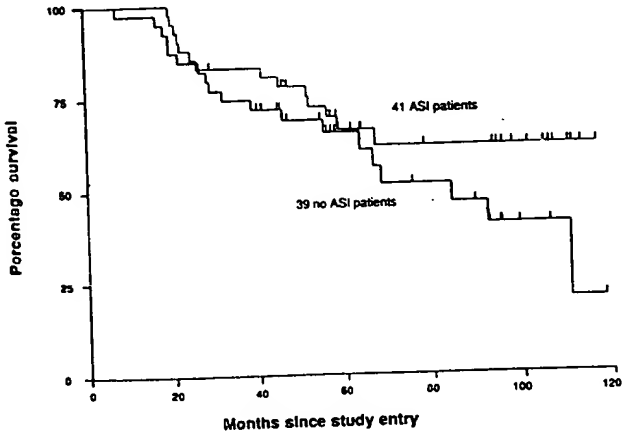

Fig 2. Kaplan-Meier curves for survival in the 80 eligible patients (colon and rectal). The hazards ratio is 1.84 in favor of ASI, but the two-sided P value is .088. (· · · · ·), ASI in all curves; ———, control.

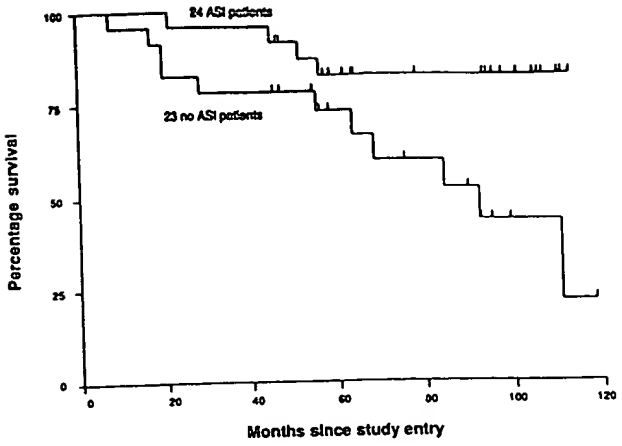

Fig 3. Distribution of months to death in 47 eligible patients with colon cancer. The hazards ratio is 3.97 in favor of ASI. The two-sided P value is .02.

of the cells. The BCG was added and mixed with the tumor cells just before injection.

The patients were observed closely after each vaccination for erythema and induration at the site of injections, fever, lymphadenopathy, or any adverse reactions. Since control patients did not receive a placebo injection, blinding of the study was not possible. With only objective end points in the study, blinding was not felt to be essential.

Both control and immunized patients were scheduled for monitoring at 3-month intervals for the first 2 years, every 4 to 6 months for the next 3 years, and once a year after 5 years. Complete blood cell counts, CEA, and tests of liver and renal function were to be performed at each visit. Physical examinations, including stool guaiacs, were also to be performed at each visit. Chest x-rays were scheduled at 6-month intervals, and liver scans and colonoscopy and/or air-contrast barium enemas were to be performed yearly. Most patients conformed well to the follow-up schedule. A documented histologic diagnosis by percutaneous or colonoscopic biopsy or reoperation was required to confirm recurrence of tumor, except in cases of lung or liver metastases with unequivocal x-ray or scan changes. Abnormal CEA levels alone were insufficient evidence for recurrence without histologic or other confirmation. The date of recurrence was listed as the date of the first abnormal test result that led to the definitive diagnosis of recurrence. At relapse, patients were candidates for surgical resection, systemic chemotherapy, or infusional chemotherapy depending on the extent and sites of recurrence.

Statistical Analysis

Statistical analysis used the Cox proportional hazards model in EGRET software (Statistics and Epidemiology Research Corporation, Seattle, WA). Informal examination of the empirical hazard function indicates the validity of the proportional hazards assumption. All *P* values are two-sided and are based on a Wald test of the treatment coefficient in a Cox model that adjusts for stage (B or C).[19] Figures 2 through 4 display the Kaplan-Meier estimates for the distribution of the survival and the disease-free survival.[20] Disease-free survival was defined as the time to a recurrence of any malignancy or death, censored at December 31, 1990 for living patients.

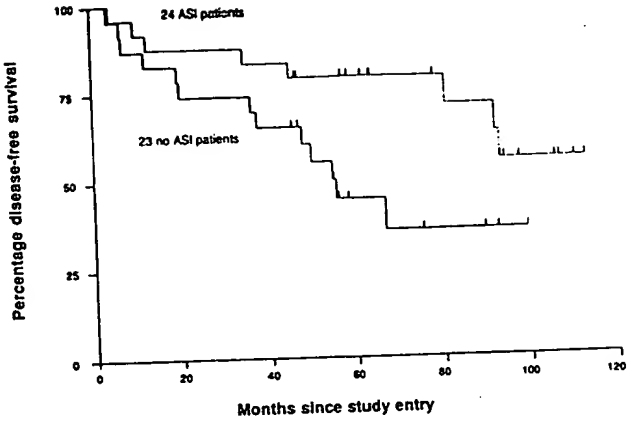

Fig 4. Distribution of months to occurrence of any malignancy or death in 47 eligible patients with colon cancer. The hazards ratio is 2.67 in favor of ASI. The two-sided *P* value is .039.

RESULTS

This report includes all patients (n = 98) who participated in the trial between March 1981 and December 1990. Every patient's record was audited by professional medical record examiners and medical oncologists and found to be accurate for all reported data using verified source documentation. Of the 98 randomized patients, the only patients not actively followed were four patients who were randomized to treatment but withdrew from the study before treatment and were lost to follow-up (Table 2). For the 94 randomized patients with complete follow-up, the median follow-up for 56 colon cancer patients is 83 months, and for 38 rectal cancer patients 57 months. For the 80 eligible patients, the median follow-up for 47 colon cancer patients is 93 months, and for 33 rectal cancer patients 58 months. No eligible patients have been lost to follow-up.

Tumor-Cell Vaccines

Tumor was dissociated and cryopreserved for 232 colorectal cancer patients viewed as potential candidates. Of these, 80 declined to participate; pathology and operative reports excluded 41 on the basis of stage (A, B_1, or D) or multiple primaries; 10 had other serious medical conditions; and technical problems with vaccine preparation excluded three patients. The remaining 98 consented to participate and were randomized.

The mean time from resection to the beginning of dissociation was 1.5 hours (range, 0.7 to 3.0), and from dissociation to cryopreservation was 5.1 hours (range, 2.5 to 9.0).

The mean weight of the tumor tissue obtained from the fresh pathology specimen was 8.3 g (range, 2.8 to 22.1). The dissociation produced a mean yield of 2.2×10^7 cells/g tumor (range, 0.5 to 10.6×10^7 cells/g). A mean prefreeze viability of 85% (range, 69% to 98%) was obtained. After thawing, the viability of the tumor cells ranged from 50% to 88%, with a mean of 77%. Within these specified viability percentages, no correlation was seen between post-thaw viability and outcome.

Patient Characteristics

Ninety-eight patients entered the trial and were randomly assigned to the immunotherapy or control groups. Four of these patients withdrew before treatment and were lost to follow-up, leaving a total of 94 patients. As listed in Table 2, 14 randomized patients have been excluded from the final statistical analysis as ineligible for the stated reasons, leaving 80 eligible patients. It should be noted that patients excluded because of advanced disease status are equally distributed between control and treatment groups (Table 2). Most of the inappropriate randomizations occurred early in the study, when patients were randomized in the immediate postoperative period of hospitalization based on the data available in the chart at that time, before the final pathology, operative, or radiology reports might show a more advanced stage. Later, patients were randomized only after all of the reports were complete. Patients were not ineligible for randomization on the basis of an elevated postoperative CEA alone, but were excluded if the CEA never returned to normal (presumed to be stage D, n = 3). We realize that excluding patients after randomization potentially detracts from the benefits conferred by randomization, especially in a small trial.[21]

The two treatment groups were remarkably similar with

Table 2. Distribution of Patients by Treatment Group

Patients	Treatment Group (no. of patients)		Total
	Treated	Control	
Enrolled (100%)	50	48	98
Excluded			
Withdrew before treatment	4	0	4
Treated in error	0	2	2
Wrong protocol (stage D)	0	1	1
Ineligible pathology (stage D)	3	3	6
Elevated (postoperative) CEA	2	1	3
Previous cancers	0	1	1
Dual primary	0	1	1
Total excluded	9	9	18
Eligible patients			80 (82%

respect to age, sex, race, location of primary tumor, pathologic stage, size of primary, extent of colon or rectal resection, and, in patients with stage C tumors, number of positive regional lymph nodes (Table 1). Five patients included as stage B_2 but with equivocal pathology findings (B_1/B_2) were equally distributed between the groups. Two were treated patients who failed to respond. Three were control patients, only one of whom failed to respond.

Protocol Violations

As explained earlier, from 1981 until 1983, patients with malignancies controlled more than 5 years earlier were considered eligible. Since 1983, patients with any prior cancer (except basal cell or squamous cell skin cancers) have been excluded. Only three patients with prior malignancies are included in the eligible study patients. None of these patients had recurrences that were related to their previous tumor. One patient had a prior early (B_1) rectal cancer that recurred in the bed of the C_3 right colon cancer for which she was entered on protocol. The second patient had a prior squamous cell carcinoma of the parotid, but died of adenocarcinoma metastatic to the lungs after entering the trial with a B_2 rectal cancer. The third patient had breast cancer treated with a mastectomy and radiation therapy 16 years earlier, but died cancer-free 5 years after entry for a C_2 colon cancer.

Protocol violations in eligible patients are listed in Table 3. These were related primarily to patients pursuing additional therapy outside of the protocol or refusing the recommended pelvic irradiation for rectal cancer. Interestingly, there have been no pelvic recurrences in the four control patients who received no pelvic irradiation or the one treated patient who received only 22 Gy. The treated patient who received four vaccines had most of the first vaccine leak from the syringe. One patient had an unusually intense reaction to her first vaccine and received no further vaccines. A second patient with an intense reaction had the BCG dose halved in the second vaccine. Two of three colon cancer patients in the ASI arm who received abdominal irradiation in violation of the protocol show no evidence of disease, while the one such patient in the control arm has recurred. Since abdominal irradiation may decrease relapse rates,[3] this imbalance of ad hoc therapy could affect the outcome in a small trial. These patients with protocol violations are all included in the analysis of eligible patients.

Immunotherapy-Related Morbidity

No serious side effects were demonstrated in the immunized patients. Since the therapy was conducted on an outpatient basis, meaningful data relative to febrile responses to the vaccine are not available. All patients were asked to keep their own temperature charts, but few recorded more than a minimal elevation of temperature in the first 2 days after receiving the first two vaccines containing BCG. As expected, all patients developed a superficial ulceration at the sites of the first and second vaccinations containing BCG. The ulcers usually occurred around the third week, were 1.5 to 2 cm in diameter, and usually healed within 3 months. Sixty percent developed palpable ipsilateral inguinal adenopathy. In each case, the adenopathy resolved within 3 months. Satellite ulcers within 5 cm of the vaccine site occurred in two patients. These resolved without any treatment. One patient had an exaggerated response to the first vaccine with induration of 15 cm and 8 cm of central ulceration. Healing occurred over a period of 3 months. She received no additional vaccines.

Liver and renal function tests were not altered by the immunotherapy. Total lymphocyte counts and absolute lymphocyte counts did not change significantly.

Table 3. Protocol Violations

Site/Stage	Violation	Current Status
Treated patients		
Colon/C_2	Abdominal RT	NED
Colon/C_2	Abdominal RT & chemotherapy	NED
Colon/C_2	Abdominal RT	Alive with hematologic malignancy
Colon/C_2	4 vaccines	Died of metastasis
Colon/B_2	1 vaccine only	NED
Colon/B_2	½ dose BCG in second vaccine	NED
Rectal/C_1	22 Gy of RT	Died of pulmonary metastasis
Rectal/C_2	RT started 8 days early	NED
Control patients		
Colon/B_2	Abdominal RT	Alive with new primary
Rectal/B_2	Refused postoperative RT	Died of pulmonary metastasis
Rectal/C_2	Chemotherapy	NED
Rectal/C_2	No RT	Died of pulmonary metastasis
Rectal/B_2	Refused postoperative RT	NED
Rectal/C_2	Refused postoperative RT	NED
Rectal/C_2	Refused postoperative RT	Alive with pulmonary metastasis

Abbreviations: RT, radiation therapy; NED, no evidence of disease.

Skin Test Reactivity

Extensive skin test data on immunized and control patients have been published.[16] Our published data on 24

immunized and 11 control patients with colorectal cancer include some patients from a stage D protocol. All patients reacted initially to at least one of the standard recall antigens. There was no change in recall antigen reactivity in the follow-up period, except that all but one of the immunized patients converted to PPD-positive. The DCH response to autologous tumor cells compared with normal mucosa cells was increased significantly ($P < .01$) in that 67% of the immunized patients showed a positive response, but only 9% of nonimmunized patients tested showed positive reactivity at the comparable postoperative period. There was not a similar boost to autologous mucosa cells in the immunized patients. In the adjuvant trial now being reported, 20 of the 41 immunized patients were tested preimmunization and postimmunization for DCH with autologous tumor cells and mucosa. Of these 20 patients, 16 (80%) became positive: 86% of colon cancer patients and 67% of rectal cancer patients.

Clinical Outcome

In our prior interim report of this trial,[17] colon and rectal carcinoma patients were analyzed together because of the small number of patients. In this report, we analyzed the total population and, recognizing the postimmunotherapy treatment difference for rectal cancer patients, performed a cohort analysis for colon and rectal carcinoma.

We considered two outcomes: survival and disease-free survival. In contrast to the 1985 interim analysis, the Cox model analysis provides no evidence of a treatment by stage interaction to support such a stratification by stage. Furthermore, the study is not powerful enough either to test the hypothesis of interaction or to compare treatment outcomes for patients separately within stages. Therefore, although the randomization was performed by stratification by stage, as well as site, the statistical decision was to view stage as dichotomous.

Table 4 lists the survival data for the 94 randomized patients (colon and rectal, eligible and ineligible, with complete follow-up), as well as for the 80 eligible patients. All analyses were adjusted for stage. Among the 94 randomized patients with complete follow-up, 46 received ASI and 48 were in the control group. Deaths from all causes were included in this analysis. In the total population of patients randomized to ASI compared with the control group, the positive trend reported in 1985[17] continues, but while the difference is impressive, it is no longer significant ($P = .068$). In the treated colon cancer patients, there was a significant survival advantage compared with controls ($P = .026$), with a hazards ratio of 2.83.

Among the 80 eligible patients, 41 received ASI and 39 were in the control group. Deaths from any cause were included in this analysis. Again, an overall positive trend is observed ($P = .088$), but the only significant survival difference ($P = .02$) was seen in the comparison of the ASI-treated colon cancer patients with the controls. The hazards ratio was 3.97.

Figures 2 and 3 show the distribution (using Kaplan-Meier[20] estimates) of survival. The Cox regression model[19] was used to compare treatment groups. Figures 2 and 3 depict the survival, respectively, for the 80 eligible patients combining colon and rectal patients and the 47 colon cancer patients.

Disease-free survival rates among the 80 eligible patients are summarized in Table 5. These analyses are expressed as disease-free survival using recurrence of any malignancy or death. This conservative analysis is of interest, as we do not know if this approach to ASI is specific for colorectal cancer or might confer broader resistance to other malignancies. When the occurrence of any malignancy or death was evaluated, there was a significant difference among the colon cancer patients ($P = .039$; hazards ratio, 2.67) (Fig 4). When the recurrence event included colon tumors only, there was a statistically significant difference between the treated and control colon cancer patients ($P = .03$; hazards ratio, 2.97).

Table 4. Summary of Survival

Patient Group	Tumor	ASI Treatment		Control		P	Hazards Ratio* (adjusted for stage)	Confidence Limits
		Deaths/Total	%	Deaths/Total	%			
Randomized (n = 94)	All	19/46	41.3	26/48	54.2	.068	1.75	—
	Colon	7/27	25.9	15/29	51.7	.026	2.83	1.13, 7.07
	Rectal	12/19	63.2	11/19	57.9	.772	1.13	—
Assessable (n = 80)	All	14/41	34.1	19/39	48.7	.088	1.84	—
	Colon	4/24	16.7	11/23	47.8	.020	3.97	1.24, 12.72
	Rectal	10/17	58.8	8/16	50.0	.930	0.95	—

*A hazards ratio > 1 favors the treatment group.

Table 5. Summary of Disease-Free Survival in 80 Eligible Patients

Type of Recurrence	Tumor	ASI Treatment Recurrence or Deaths/ Total	ASI Treatment %	Control Recurrence or Deaths/ Total	Control %	P	Hazards Ratio* (adjusted for stage)	Confidence Limits
Any malignancy or death	All	19/41	46.3	23/39	58.9	.147	1.58	—
	Colon	8/24	33.3	13/23	56.5	.039	2.67	1.05, 6.76
	Rectal	11/17	64.7	10/16	62.5	.905	1.05	—
Primary or second colon tumor or death	All	17/41	41.5	23/39	58.9	.112	1.67	—
	Colon	6/24	25.0	13/23	56.5	.030	2.97	1.11, 7.94
	Rectal	11/17	64.7	10/16	62.5	.905	1.05	—

*A hazards ratio > 1 favors the treatment group.

In Figs 3 and 4, it is apparent that late events (> 60 months) are important in survival and disease-free survival. The three late events in the treated group involve diagnoses of malignancies unrelated to the primary tumor (a second primary colon cancer and two hematologic malignancies). In the control group, three of four late events were due to progression of the primary tumor and one was death due to a new primary tumor (renal cancer).

DISCUSSION

This report updates our previously published, prospectively randomized, controlled trial of ASI in colon and rectal cancer.[17] A statistical analysis of this small trial suggests therapeutic benefit from autologous immunization in patients with colon but not rectal cancer, although with such small numbers of patients we cannot conclude that ASI is of proven therapeutic benefit.

That colon cancer patients appear to benefit while rectal cancer patients do not is of considerable interest. It could be that rectal cancers are intrinsically less immunogenic. We do not have sufficient data to confirm that hypothesis on the basis of DCH reactivity. We are concerned that the lack of ASI effect in rectal cancer patients could be related to the logistics of the pelvic irradiation. In the guinea pig model, the draining lymph nodes must be left intact for 21 days following immunization to develop sufficient immunity to destroy tumor. Rectal cancer patients started pelvic irradiation, including the draining lymph nodes, within a few days after completing immunization. It is possible that the lymph nodes targeted by immunotherapy were destroyed by the irradiation before they could have a full impact on the immunologic response.

We now have three lines of evidence that ASI has an immunologic impact in patients with colon cancer: (1) we have previously reported a significant boost in reactivity (48-hour DCH) to autologous tumor cells in vaccinated patients[16]; (2) we have reported the use of peripheral-blood lymphocytes from these immunized patients as sources for the development of stable clones of human B lymphocytes that produce colon and rectal tumor-specific monoclonal antibodies[22] (lymphocytes harvested from the peripheral blood of unimmunized patients are less likely to contain cells that produce tumor-specific monoclonal antibodies); and (3) the sustained differences in clinical outcome between immunized and nonimmunized groups of patients with colon cancer in our study are encouraging, even with the small numbers.

How can we explain our modest gains in the face of so many past failures of attempts to control malignant disease by immune manipulations? A reappraisal of the past failures in the light of current studies in experimental animal models may clarify the reason for the failures and suggest other new immunologic approaches. Our clinical protocol emerged from biologic principles derived from nearly 8 years of investigation with the guinea pig line-10 hepatocarcinoma model. Studies in the guinea pig model demonstrated that BCG mixed with tumor cells is effective in inducing a degree of systemic tumor immunity capable of eliminating a limited disseminated tumor burden. Further studies demonstrated the feasibility of preparing tumor-cell suspensions from enzymatically dissociated solid tumors without loss of immunogenicity, a requirement for the preparation of human tumor vaccines.[14] It was possible in this model to demonstrate that allogeneic cells, dead cells, or cell components (antigen extracts) were not effective. It also was demonstrated that BCG components were not effective substitutes for whole viable BCG cells. Autologous tumor cells alone or BCG alone were not effective.

Past attempts at controlling colon cancer by immunologic means have used primarily nonspecific immunomodulators, none of which have significantly altered the course of the disease. Interestingly, the National Surgical Adjuvant Breast and Bowel Project protocol C-01 reported a survival advantage in favor of BCG scarification

treatment over resection alone in patients with Dukes' stage B and C carcinoma of the colon.[23] However, further investigation disclosed that the survival advantage in favor of BCG was a result of a diminution in non–cancer-related deaths. Deaths from cardiovascular-related sequelae were twice as common in the untreated control group as in the BCG-treated cohort. In the present study, there were two non–cancer-related deaths, one in the control colon cancer group and one in the ASI-treated colon cancer group.

Most human trials with tumor vaccines have had at least one major variation from the requirements listed above for successful immune stimulation with ASI. Most have used tumor cells of low viability, an absolute predictor of failure in our model. Such cells disintegrate rapidly and do not provide the necessary sustained antigenic stimulus to the regional lymph nodes. Others have used allogeneic cell lines. Unless cross-reacting tumor antigens are shared by the donor and recipient, allogeneic vaccines are destined to fail.

In the guinea pig model, the success of therapy is inversely proportional to the number, size, and location of metastases present at the time of treatment. It is unlikely that even the most optimal vaccination regimen could control large tumor burdens. Studies in the guinea pig model have suggested that ASI can become a meaningful, integral part of combination therapy, when coupled with conventional chemotherapy or additional biologic therapy.[24] Studies have shown that chemotherapy acts synergistically with ASI in the eradication of pulmonary micrometastases if a precise sequence of immunotherapy and chemotherapy is followed.[25,26] The mechanism for this synergistic interaction appears to be an immune-mediated breakdown of the normal anatomic and vascular barriers in solid tumors, allowing greater access of blood-borne chemotherapeutic agents to the tumor cells.

We are attempting to verify the potential efficacy of ASI therapy through prospectively randomized studies underway under the auspices of the ECOG using ASI in an adjuvant setting in Dukes' stage B and C colon cancer patients. The Dukes' stage C study evaluating ASI alone has been closed with adequate accrual for reliable assessment and accrual continues with the Dukes' stage B colon patients. A second phase III trial has been initiated combining immunotherapy and chemotherapy in patients with advanced disease. With the recently reported benefits from adjuvant 5-FU and levamisole,[6,7] our adjuvant protocols have been altered to include 5-FU and levamisole in all Dukes' stage C colon cancer patients. This will give an optimal opportunity to determine the efficacy of ASI in combination with chemotherapy in the adjuvant setting.

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REFERENCES

1. Boring CC, Squire TS, Tong T: Cancer statistics, 1991. CA 41: 19-36, 1991

2. Tepper JE: Radiation therapy of colorectal cancer. Cancer 51: 2528-2534, 1983

3. Willett CG, Tepper JE, Skates SJ, et al: Adjuvant postoperative radiation therapy for colonic carcinoma. Ann Surg 206:694-698, 1987

4. Gastrointestinal Tumor Study Group: Prolongation of the disease-free interval in surgically treated rectal carcinoma. N Engl J Med 312:1465-1472, 1985

5. Laurie JA, Moertel CG, Fleming TR, et al: Surgical adjuvant therapy of large bowel carcinoma: An evaluation of levamisole and the combination of levamisole and fluorouracil. J Clin Oncol 7:1447-1456, 1989

6. Moertel CG, Fleming TR, MacDonald JS, et al: Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma. N Engl J Med 322:352-358, 1990

7. Krook JE, Moertel CG, Gunderson LL, et al: Effective surgical adjuvant therapy for high-risk rectal carcinoma. N Engl J Med 324: 709-715, 1991

8. Kennett RH, Jonak ZL, Bectol KB: Monoclonal antibodies against human tumor-associated antigens, in Kennett RH, McKearn TJ, Bechtol KB (eds): Monoclonal Antibodies: Hybridomas: A New Dimension in Biological Analyses. New York, NY, Plenum, 1980, pp 55-168

9. Hanna MG Jr, Brandhorst JS, Peters LC: Active specific immunotherapy of residual micrometastasis: An evaluation of sources, doses and ratios of BCG with tumor cells. Cancer Immunol Immunother 7:165-173, 1979

10. Hanna MG Jr, Peters LC: Specific immunotherapy of established visceral micrometastases by BCG-tumor cell vaccine alone or as an adjunct to surgery. Cancer 42:2613-2625, 1978

11. Hanna MG Jr, Pollack VA, Peters LC, et al: Active-specific immunotherapy of established micrometastases with BCG plus tumor cell vaccines: Effective treatment of BCG side effects with isoniazid. Cancer 49:659-664, 1982

12. Hoover HC Jr, Peters LC, Brandhorst JS, et al: Therapy of spontaneous metastases with an autologous tumor vaccine in a guinea pig model. J Surg Res 30:409-415, 1981